MEETING

SCIENTIFIC ADVISORY BOARD

October 30, 31, November 1, 1974

H Walsehan

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CARDIOVASCULAR

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#1010 - BECKER

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 19, 1974

Grant application No. 1010

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Feldman, Liebow

Subject: Carl G. Becker, M.D., Cornell University Medical College
New application No. 1010
"Investigation of the Role of Allergy to Tobacco Constituents in the Pathogenesis of Arteriosclerosis and Myocardial Infarction".

History

Initially Dr. Hockett visited George E. Murphy, M.D. with Alexander Holtzman, Esq., of Philip Morris Inc. Subsequently, before the application was prepared, a formal site visit was conducted by Drs. Bing, Hockett and Sommers. Enclosed is Dr. Bing's letter dated August 5, 1974 commenting on the visit.

Request

Application No. 1010 requests $101,888 plus two additional years.

Documents Submitted (attached)

1. Application dated August 12, 1974 (Figures 1 and 2, in color, of course do not Xerox clearly; the originals will be loaned to you if you wish and will, in any event, be brought to the SAB meeting).

2. C.V.'s of Drs. Becker, Levi, Minick and Murphy.

Comment

Dr. Hockett may send you a written commentary in due course.

F.W.N.

FWN:wg
Encls.

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1010

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
AUG 16 1974

Application for Research Grant
(Use extra pages as needed)

Date: August 12, 1974

1. Principal Investigator (give title and degrees):

Carl G. Becker, M.D., Associate Professor of Pathology, Principal Investigator
Roberto Levi, M.D., Associate Professor of Pathology, Co-investigator
C. Richard Minick, M.D., Assoc. Professor of Pathology, Co-investigator
George E. Murphy, M.D. Professor of Pathology, Co-investigator

2. Institution & address:

Cornell University Medical College
Department of Pathology
1300 York Avenue
New York, New York 10021

3. Department(s) where research will be done or collaboration provided:

Departments of Pathology and Pharmacology

4. Short title of study:

Investigation of the Role of Allergy to Tobacco Constituents in the Pathogenesis of Arteriosclerosis and Myocardial Infarction.

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

Smoking of tobacco and atherosclerosis have been associated statistically (1-4). The pathogenetic mechanisms that are the basis of this relationship are obscure. Research proposed herein is designed to examine the hypothesis that certain people become allergic to constituents of tobacco or tobacco smoke and that injury to blood vessels, arteriosclerosis, and injury to the myocardium may be consequent to repeated challenge with tobacco allergens (5-7). Scarring of pulmonary blood vessels has been observed with greater frequency in lungs of cigarette smokers than in lungs of pipe or cigar smokers or non-smokers (8).

In the course of these investigations we propose to:

a) Isolate, purify and identify as allergenic, material from tobacco. We propose to identify the route of entry and absorption of this allergenic material.

b) Examine sera from non-smokers and smokers with and without clinical evidence of ischemic heart disease and with and without pulmonary emphysema for the presence of reaginic (IgE) antibodies

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Specific research aims (cont'd)

to tobacco antigens by radioimmunoassay technique. We propose to skin test the same subjects for immediate and delayed hypersensitivity to these antigens. Because of recent data indicating that in rabbits production of anaphylactic or IgE antibodies can be especially stimulated by neonatal immunization, we propose to examine the sera of children from smoking and non-smoking mothers for IgE antibodies to tobacco antigens. If possible, we shall also skin test these children.

c) Purified constituents of tobacco which we identify as allergenic in human smokers and the route(s) of absorption of which we determine will be used to sensitize rabbits. The character of the immunologic response of rabbits to these antigens will be determined. Sensitized rabbits will be fed a lipid rich diet and repeatedly challenged with tobacco allergens by the same route by which humans are exposed to these allergens to test the hypothesis that tobacco induced allergic injury to blood vessels and hyperlipidemia may act synergistically in the development of athero-arteriosclerosis.

d) Rabbits sensitized to tobacco allergen(s) will be challenged with these allergens by appropriate route and observed for signs and symptons of cardiac anaphylaxis in vivo. The hearts of some sensitized rabbits will be challenged in vitro with the appropriate antigen and observed for the development of cardiac anaphylaxis.

8. Brief Statement of Working Hypothesis:

The hypothesis underlying the proposed research is that some tobacco smokers become sensitized to one or more allergenic components of tobacco smoke and that repeated challenge with these allergens by smoking can result in immunologic injury to blood vessels, accentuating the development of atherosclerosis and increasing the risk of myocardial infarction. Similarly, repeated immunologic injury to pulmonary blood vessels may contribute to the development of pulmonary emphysema. A related, additional hypothesis is that some smokers sensitized to tobacco allergens can develop myocardial anaphylaxis on repeated contact with these allergens.

9. Details of Experimental Design:

A. Purification and Identification of Tobacco Allergens:

1) Tobacco Leaf Glycoprotein (TGP) -

We have extracted cured tobacco leaves of Maryland, Turkish, Burley, and Flue cured varieties (obtained through the courtesy of American Tobacco Co.) with phosphate buffered

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Details of experimental design (cont'd)

saline, pH 7.4 (PBS) after delipidization with petroleum ether. The saline extracts were brought to 60% saturation with crystalline $(NH_4)_2SO_4$, redissolved in PBS and applied to G-25 Sephadex columns from which it emerged with the void volume as a single, sharp, symmetrical peak. When this peak was applied to 7.5% polyacrylamide gels, pH 8.9 and subjected to electrophoresis it migrated rapidly and anodally as a single, brown band which stained with both protein stains (Amido black or Coomassie blue) and the PAS reaction, indicating that it is a glycoprotein (Fig. 1).

The electrophoretic migration and staining characteristics of material extracted from the four different kinds of tobacco were identical (Fig. 2). This purified material, when injected intradermally induced wheal and flare reactions within 30 minutes of intradermal injection in three of six smokers and in one non-smoker with a history of rhinorrhea in response to tobacco smoke exposure. It produced no reaction in two of two non-smokers. This material is presumably the allergenic principle present in tobacco extracts described by Harkavy and Perlman (5). For purposes outlined in this research proposal tobacco leaf glycoprotein antigen (TGP) will be prepared as outlined above.

2) Tobacco Leaf Lipids -

a) Rhus toxin is volatilized when leaves of the genus Rhus (poison ivy, poison oak, poison sumac) are burned, yet still capable of inducing contact dermatitis by mechanisms related to delayed hypersensitivity in sensitive subjects. We propose to examine the lipid soluble material from cured tobacco leaves for allergenic material that on burning become volatilizable haptens.

b) The petroleum ether extract of cured tobacco leaves that is obtained from the first stage of preparation of TGP will be concentrated by evaporation of the petroleum ether. The resulting material will be assayed for allergenicity as described below.

B. Demonstration of Hypersensitivity to Tobacco Leaf Glyco-Protein (TGP) and Characterization of Antibodies to TGP in Human Serum:

1) TGP, in doses of 10 and 50 µgrams will be used to test for immediate and delayed hypersensitivity to TGP by intradermal injection in

a) smokers without evidence of ischemic heart disease,

b) age-matched smokers with evidence of ischemic heart disease,

c) age-matched non-smokers,

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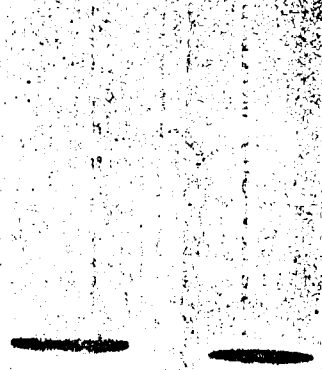
Fig. 1



Turkish tobacco protein

(from left to right)
Unstained, Coomassie blue, PAS

Fig. 2

PAS

(from left to right)
Burley, Maryland, Turkish, Flue cured

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Details of experimental design (cont'd)

d) patients with thromboangiitis obliterans

e) smokers with clinical evidence of emphysema

f) non-smokers with clinical evidence of emphysema or other chronic pulmonary disease

g) smoking and non-smoking medical students

h) former smokers who have given up smoking.

The injection sites will be observed for wheal and flare reactions and for the development after 24 hours of the indurated lesion characteristic of delayed hypersensitivity. Persons tested will be questioned for history of allergy. A serum sample will be obtained from all persons tested.

2. Characterization of the antibodies to TGP.

a) TGP will be coupled to Sepharose beads activated with cyanogen bromide to prepare an insoluble immunoabsorbent (S-TGP) (9).

b) Aliquots of serum from subjects described above will be incubated with S-TGP and then centrifuged and washed several times.

c) After step (b), aliquots of S-TGP will then be incubated with I^{125}-labeled antibodies specific for heavy chains of IgE, IgG, IgA and IgM. After centrifugation and washing, the radioactivity will be counted and the quantity of immunoglobulin specifically bound to S-TGP calculated from previously prepared standard curves.

d) Data obtained by this radioimmunoassay will provide information concerning (i) the nature of the humoral antibody response to TGP and (ii) the frequency of people with antibodies to TGP.

Skin testing for immediate hypersensitivity alone would not give an accurate measure of the frequency of immunization against TGP since it reflects predominantly the presence or absence of reaginic or IgE antibodies.

3. Characterization of the route of absorption of TGP.

a) The question arises as to how TGP is absorbed. Is a hapten released from it when tobacco is burned? Or, is TGP taken in with steam generated behind burning tobacco? In this connection, an allergenic muscle protein has been demonstrated in the steam from cooking codfish (10). We propose to examine saliva collected from smoking subjects and condensates of tobacco smoke for the presence of intact

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Details of experimental design (cont'd)

TGP or fragments of TGP present as haptens. We also propose to examine the blood of smokers for TGP antigen. A radioimmunoassay using I^{125}-labeled TGP and competitive protein binding technique will be the means of these measurements.

b) Antisera to TGP will be prepared in rabbits. Antibody to TGP will be purified from sera from immunized rabbits using columns of TGP coupled to Sepharose.

c) TGP will be radio-labeled and the capacity of standard anti-TGP sera to bind TGP determined. Antibody will then be incubated with known amounts of cold TGP and then with labeled TGP and a standard curve constructed.

d) Saliva and blood of subjects will be collected before, during, and after smoking. We will look for TGP in saliva and serum samples by measuring the capacity of the samples to inhibit binding of radio-labeled TGP to anti-TGP.

e) If there is evidence of TGP in saliva, a large quantity of saliva will be collected from smoking subjects. In order to see if TGP is present in intact form we shall attempt to reisolate it from saliva and compare it physically and immunochemically with TGP isolated from tobacco leaves.

f) Failure to reisolate intact TGP from saliva containing TGP reactive with anti-TGP would suggest that it is present as a hapten. If so, we shall attempt to isolate the hapten by using anti-TGP coupled to an insoluble matrix.

g) We also propose to examine condensates of tobacco smoke for TGP by radioimmunoassay as well as attempt to isolate intact and/or haptenic TGP from these condensates.

C. Assay of Tobacco Leaf Lipid (TLL) for Allergenic Activity:

1) It is not known whether any of the lipid soluble material in tobacco leaf is allergenic. In fact, in two recent publications dealing with the chemical nature of allergens, there is no mention of allergenic lipids (11,12).

2) The crude lipid extract will be prepared as described above. Since this is a complex mixture of unknown properties we will assay it for allergenicity by indirect means rather than by skin testing human subjects.

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Details of experimental design (cont'd)

3) Some subjects allergic to tobacco glycoprotein (TGP) might well be allergic to TLL as well. Serum from subjects with high levels of IgE antibodies to TGP and serum from subjects with no antibodies to TGP will be injected intradermally into a Rhesus monkey. After 50 hours and immediately following systemic injection of Coomassie blue, the sites of serum injection will be injected with TLL (Prausnitz-Kustner (P-K) reaction) and observed for blueing. Alternatively, the TLL will be injected intravenously and the sites of serum injection observed for blueing (Passive Cutaneous Anaphylaxis (PCA) reaction. The TLL may not be soluble in saline, in which case we would extract TLL with the monkey's own serum in the hope that some of the lipid components might bind to serum albumin and/or lipoproteins. The TLL serum extract would then be used for P-K or PCA reactions.

4) Blood will be drawn from subjects with and without reaginic antibodies to TGP and the capacity of TLL to stimulate histamine release from their leukocytes measured (13).

5) If the lipid extracts are shown to have allergenic activity, we propose to fractionate the lipid extract to isolate the specific allergen(s) using techniques of thin layer chromatography on silica gels, solvent resistant Sephadex, etc.

6) Lipid fractions obtained in paragraph (5) would be assayed as in paragraphs (3) and (4) for allergenic activity.

7) Purified lipid fractions demonstrated to have allergenic activity will be coupled to insoluble matrices and human sera will be examined for antibodies to them by radioimmunoassay technique as described above for TGP. If the lipid fractions are nontoxic we would also attempt skin testing of human subjects.

8) If it can be shown that certain lipid components of tobacco leaf are allergenic we would like to examine condensates of tobacco smoke for their presence, employing techniques of thin layer chromatography or gas liquid chromatography. Lipids recovered from tobacco smoke and corresponding chromatographically to those isolated from tobacco leaf will be assayed for allergenicity as described above.

D. Studies on the Role of Allergy to Tobacco in the Pathogenesis of Athero-arteriosclerosis and Pulmonary Emphysema:

In preceding sections of this proposal we have described the purification of one component of tobacco leaf that is allergenic in humans and described how we plan to look for

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Details of experimental design (cont'd)

others. However, the identification of tobacco allergens and even the possible demonstration of an association between allergy to tobacco and arteriosclerosis would only fulfill the first two of the Koch-Henle postulates. It is necessary to sensitize experimental animals to these allergens and by repeatedly exposing sensitized animals to these allergens by appropriate route of administration induce arteriosclerosis. To fulfill the fourth of these postulates it would also be necessary to recover the allergens from the vascular lesions. This last may not technically be possible because of the small amounts of allergen involved.

In previous experiments we have shown that the synergy of modest hypercholesterolemia (200-250 mg%) and immunologic injury induced by repeated injection of foreign serum induced chronic coronary atherosclerosis in rabbits that closely resembles coronary atherosclerosis in man (14,15,16). We have also shown that endothelium of coronary and other arteries contains contractile protein and proteins regulatory of contraction (17,18,19). The endothelium is therefore a dynamic rather than passive lining. Stimulation of endothelial contraction may result in enhanced permeability of the artery wall to macromolecules such as β-lipoprotein and immune aggregates. In this connection, it has been demonstrated that vasoactive amines (histamine and serotonin) enhance deposition of immune aggregates. It has also been demonstrated that stimulation of reagin (IgE) coated basophiles with specific antigen not only results in release of their vasoactive amines but also release of a different substance which causes platelets to release their vasoactive amines enhancing immune complex deposition (20).

Taken together, these data certainly suggest that chronic repeated exposure of a sensitized subject to specific allergen might result in chronic injury to blood vessels and the development of arteriosclerosis. If similar chronic injury occurs in pulmonary vessels, it might result in focal destruction of pulmonary parenchyma and emphysema.

We propose to test this hypothesis in the following way:

1) Young male rabbits will be divided into three groups. All three groups will be sensitized with the tobacco allergen to which human smokers most consistently can be demonstrated to be allergic. In order to sensitize as many rabbits as possible the insolubilized antigen will be injected in foot pads. The sera of injected rabbits will be assayed for the presence of 1) reaginic antibodies by a) direct skin testing, and b) capacity to induce PCA reactions in other rabbits; and 2) other humoral antibodies by immunodiffusion and radioimmunoassay.

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Details of experimental design (cont'd)

The rabbits will be tested for delayed hypersensitivity to tobacco antigen by skin testing. If delayed hypersensitivity is frequently exhibited, we will attempt to confirm its presence in vitro by measuring inhibition of leukocyte migration with tobacco antigen. Rabbits capable of being sensitized with respect to immediate hypersensitivity will be divided into three groups.

2) Rabbits in group I and III will be started on atherogenic diet. This diet approximates the average American diet with respect to the number of calories supplied as fat, carbohydrate and protein as defined by Stammler, et al (21). The rabbits will be fed this diet throughout the experimental period.

3) Rabbits in group I will be diet controls. Rabbits in groups II and III will be repeatedly exposed to tobacco allergen by the same route as the antigen is absorbed in humans and in proportional quantity. If it is shown that the principle route of absorption of antigen in humans is smoke, then sensitized rabbits will need to be exposed to smoke. If antigen is taken into saliva and absorbed, then antigen can be fed to the rabbits. We feel that to be a meaningful experimental model, both route and dosage of antigen should be the same as occurs in human smokers. It is known that with certain doses of intravenously administered horse serum or bovine serum albumin arterial lesions can be induced, but these models are probably not analogous to what occurs in the smoking subject.

4) During the period of the experiments, rabbits will be bled and skin tested at frequent intervals in order to measure any change in their immunological response to tobacco antigen.

5) Rabbits of all three groups will be sacrificed after 6-9 months. Tissues will be fixed, and sectioned, and atherosclerosis will be quantified as described previously (14).

6) One lung from each rabbit will be fixed and sectioned in an expanded state as described by Sills in order to evaluate emphysematous change (22). Multiple sections of the contralateral lung will be examined histologically.

7) Data will be analyzed with respect to: a) whether repeated immunologic challenge with tobacco allergen of rabbits sensitized to tobacco allergen leads, in the presence of modest hyperlipidemia, to the development of athero-arteriosclerosis; b) whether repeated challenge of rabbits sensitized to tobacco results in injury to the pulmonary vasculature and,

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Details of experimental design (cont'd)

in turn, to the development of emphysema; c) which component of the immune response correlates most closely with the development of systemic arteriosclerosis and/or pulmonary emphysema, i.e. the quantity of reaginic antibody produced, the quantity of other antibodies produced, or whether or not delayed hypersensitivity to tobacco antigen develops.

E. Studies on the Role of Atopic Injury Due to Tobacco Antigens and the Pathogenesis of Athero-arteriosclerosis:

The objective of this experiment is to test the hypothesis that atopic or IgE mediated responses to exogenous antigen may act synergistically with hyperlipidemia to induce athero-arteriosclerosis.

It has been shown in this laboratory that allergic injury to blood vessels probably mediated by deposition of circulating immune complexes acts synergistically with hyperlipidemia to produce arterial lesions which closely resemble athero-arteriosclerosis in man. Moreover, we have observed accentuated or precocious athero-arteriosclerosis in coronary and other arteries in young patients with lupus erythematosus, also an immune complex disease, and in some young patients with rheumatic heart disease. However, a relatively few humans have immune complex disease. On the other hand, many people display atopic or IgE mediated allergy to a large variety of exogenous antigens. The mechanism of these IgE mediated responses involves the release of factors which increase blood vessel permeability perhaps by stimulating contraction of endothelial cells, which we have shown to contain contractile and regulatory proteins.

Pinckard, et al observed that immunization of day old rabbits with appropriate doses of alum precipitated bovine serum albumin (BSA) could lead to the preferential synthesis of IgE type antibodies to BSA and apparent tolerance to BSA with respect to the production of other immunoglobulins (21). We have tried to reproduce these observations. We found that when New Zealand red rabbits or Chinchilla rabbits are immunized on the first day of life by intraperitoneal injection with 0.25 - 0.5 mgs of alum precipitated bovine serum albumin (BSA) and subsequently boostered at monthly intervals with BSA that some of these rabbits selectively produce homocytotropic heat-labile antibodies as demonstrated by passive cutaneous anaphylaxis. Litter-mates immunized a week to 10 days after birth produced precipitating antibodies and rapidly cleared radio-labeled BSA from their circulation. When injected intravenously with as little as 100 µgms of soluble BSA, rabbits with homocytotropic antibody were exquisitely sensitive to anaphylaxis. This anaphylaxis is cardiovascular rather than

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Details of experimental design (cont'd)

pulmonary and the rabbits develop cardiac arhythmias and hypotension. This subject will be taken up again later. The mean arterial pressure may fall from 100 mm Hg to 40 mm Hg for as long as 20 minutes and some of these animals die. By reducing the antigenic challenge dose death by anaphylaxis can be avoided but periods of enhanced blood vessel permeability perhaps obtained. If one applies this observation to the construction of a hypothesis concerning events which may take place in the sensitized human smoker it is conceivable that repeated challenge through the course of the day of the atopic smoker with tobacco antigen results in prolonged change in vessel permeability to macromolecules such as lipoproteins. This may lead to the accentuation of the development of arteriosclerosis. If cardiac anaphylaxis occurs in humans as it does in the rabbit, it may also lead to the development of myocardial infarct by producing cardiac arhythmias and hypotension in the presence of already narrowed coronary blood vessels. Another hypothesis is easily constructed from this data. Mothers who smoke may conceivably sensitize their children in utero or neonataly in such a way that they are conditioned to produce reaginic or IgE type antibodies to tobacco constituents. It may be that the group of smokers who are most at risk with respect to the development of ischemic heart disease are at risk because of events that took place during intrauterine life. We propose to test the hypothesis that atopic or IgE mediated sensitivity to tobacco constituents may lead to arteriosclerosis and that neonatal sensitization with this antigen contributes to the development of the atopic state.

1) Random stock New Zealand red or Chinchilla rabbits will be mated.

2) Litters of these matings will be divided into two groups. Half of the young will be injected on the first day of life with an appropriate dose of tobacco antigen with corynebacterium parvum as adjuvant. These young rabbits will be boostered at appropriate intervals thereafter. The rest of the litter will not be injected.

3) These rabbits will be bled and serum tested for heat-labile, 2-mercaptoethanol sensitive, homocytotropic antibody to tobacco antigen by passive cutaneous anaphylaxis and precipitating antibodies to tobacco antigen by immunodiffusion or possibly by radioimmunoassay employing tobacco antigen bound to an insoluble matrix and radio-labeled antibodies to immunoglobulins other than IgE.

4) It will be established that some rabbits have either a) only IgE antibodies to tobacco antigen, or b) no antibodies to tobacco antigen.

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Details of experimental design (cont'd)

pulmonary and the rabbits develop cardiac anaphylaxis and

Rabbits falling into these classes will then be equally and repeatedly exposed to tobacco antigen by doses and by routes demonstrated to be those which pertain in human smokers. Half of these rabbits will also be fed the atherogenic diet described in the previous section. Diet controls in experiments described in the previous section will serve as diet controls for this experiment also.

which may take place in the sensitized human smoker it is

7) After 9 months of repeated exposure to tobacco antigen rabbits in all groups will be sacrificed and tissue fixed and sectioned as described previously. The lungs will also be fixed and examined as described in the previous section.

the development of arteriosclerosis. If cardiac anaphylaxis

8) During the course of these experiments rabbits will be bled repeatedly in order to discern any change in the nature of their immune response to tobacco antigens.

9) Data will be compared to determine if atopic or IgE mediated responses to repeated contacts with tobacco antigen leads to the development of systemic arteriosclerosis and/or development of pulmonary emphysema. These data will be important to determining if the atopic smoker is at special risk. Identification of atopic smokers and the removal of tobacco from such smokers may be important to reducing their risk of arteriosclerosis, ischemic heart disease and possibly pulmonary emphysema.

F. Studies on the Role of Tobacco Allergens in the Development of Cardiac Anaphylaxis:

The purpose of the research proposed in this portion of this application is to

a) investigate the cardiovascular manifestations of antibody-mediated adverse reactions to tobacco antigens,

b) define the role played by histamine and to ascertain its relative contribution to cardiac hypersensitivity reactions to tobacco antigens,

c) explore means of pharmacological intervention.

Clinical manifestations of acute hypersensitivity reactions in man include respiratory distress and cardiovascular collapse (24). Cardiovascular collapse may be secondary to the asphyxiating effects of bronchospasm and laryngeal edema. Acute circulatory failure, however, may occur without preceding respiratory distress (24,25) and stem from a ventricular arrhythmia or an acute myocardial infarction (26,27,28, 29,30,31). Recently, Delage, et al (32) reported histochem-

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Details of experimental design (cont'd)

ical evidence of early myocardial damage in sections from 24 out of 30 patients who died of drug-induced anaphylactic shock. These lesions were interpreted as the possible result of an immunologic reaction on the myofibers or as an effect of the pharmacologic mediators of anaphylaxis.

The capacity of the heart to be involved in hypersensitivity reactions has been definitively demonstrated in vitro in the guinea pig. Furthermore, electrocardiographic changes have been observed during systemic anaphylaxis in experimental animals and in humans. Following systemic anaphylaxis characteristic lesions have been observed in hearts of animals and humans. Therefore, the involvement of the heart in human acute allergic reactions is highly probable. The extent and implications of this must be evaluated in the experimental animal as a first step in the assessment of cardiac hypersensitivity as a clinical problem. Since the pattern of anaphylactic response is species-dependent, and since similarities exist between the IgE of humans and rabbits, these should be regarded as an ideal experimental model. We will attempt to delineate cardiovascular manifestations of immediate hypersensitivity reactions in the rabbit. Having done so, we will attempt to delineate pharmacologic means of alleviating cardiac dysfunctions caused by immunologically mediated release of histamine.

1) Participation of the heart in tobacco allergy:

a) Rabbits will be sensitized by injection into foot pads in complete Freund's adjuvant of tobacco components demonstrated to be allergenic in man. If, in previous experiments, it is observed that injection of the neonatal rabbit with tobacco allergen results in a high incidence of animals producing reaginic antibody, then we shall prepare rabbits in experiments proposed here by neonatal sensitization.

b) The production of a reaginic-type antibody will be checked by the persistence of the PCA (passive cutaneous anaphylaxis) reaction after 7 days and by heat-lability and mercaptoethanol sensitivity of the antibody responsible for the PCA reaction.

c) Hearts will then be isolated, perfused in a Langendorff apparatus, and challenged by intra-aortic injection of antigen. The effect of the challenge on rate, ventricular contraction, atrioventricular conduction, rhythmicity and coronary flow will be observed. Coronary effluents will be assayed for the presence of histamine (33,34).

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Details of experimental design (cont'd)

d) Subsequently, the relative contribution of histamine to tobacco-induced cardiac anaphylaxis will be studied on the isolated heart and the isolated coronary artery strip. Drugs capable of depleting, or blocking the actions of histamine will be used in this connection. Depletion will be attempted with 48/80 prior to antigenic challenge; blockade will be induced with a combination of chlorpheniramine (anti-H_1 antihistamine) and burimamide (anti-H_2 antihistamine). This combination should inhibit all of the cardiac actions of histamine (35).

2. Studies on anaphylaxis of coronary arteries induced in vitro by tobacco antigen:

a) Dogs and/or Rhesus or African Green Monkeys will be sensitized with tobacco components demonstrated to be allergenic in man.

b) Excised coronary arteries will be isolated and mounted as helical strips according to the technique of Zuberbuhler and Bohr (36). Tobacco allergens will be added to the bathing fluid and changes in tension will be monitored.

3. Pharmacologic intervention in tobacco-induced anaphylaxis:

a) The initial step in the search for drugs capable of interfering with anaphylaxis of the heart will be to establish an in vitro model of graded anaphylaxis to tobacco antigens. A graded reaction will allow pharmacological quantitation and manipulation on the basis of dose-response relationships. It is expected that such a model will be obtained by passive sensitization in vitro (33, 37). Reaginic antisera against tobacco antigens will be prepared in the rabbit. Hearts from normal rabbits will be perfused with varying amounts of rabbit antiserum and challenged with constant doses of antigen. This procedure will allow the construction of a curve relating amounts of sensitizing antibody to amount of cardiac histamine release, with histamine functioning as an indicator of cardiac anaphylaxis (33). Pharmacological manipulations will be conducted in hearts sensitized with antibody concentrations chosen from the linear portion of this curve. By this procedure it will be possible either to change the dose of an inhibi-

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Details of experimental design (cont'd)

tory drug while maintaining constant the level of sensitization or conversely to study the effects of a single drug dose on a range of anaphylactic responses.

b) Glucagon and theophylline, through increase of intracellular cyclic AMP, are capable of inhibiting the immunologic release of histamine. These will be tried first, since they were shown by one of the applicants to inhibit the anaphylactic ovalbumin-induced release of histamine from the isolated guinea pig heart (38,39).

c) Among the possible physiological antagonists of cardiac anaphylaxis epinephrine will be investigated, not only because of its capacity to increase the intracellular levels of cyclic AMP, but mostly because epinephrine has been traditionally the drug of choice in the treatment of anaphylaxis. Although epinephrine may be expected to counteract the detrimental effects of anaphylaxis on atrioventricular conduction, epinephrine will also add to the positive chronotropic effect of histamine and to the increase in ventricular automaticity. It is, therefore, imperative that the antianaphylactic effects of epinephrine be reinvestigated in connection with the cardiac effects of anaphylaxis to tobacco antigens. Besides epinephrine, another possible physiological antagonist, diphenylhydantoin, will be used because of its known beneficial effect on the restoration of atrioventricular conduction and the suppression of ventricular arhythmias (40).

d) Specific antagonism of cardiac anaphylaxis will be tried with a combination of antihistamines. It is likely that a combination of H_1- and H_2-receptor blockers will greatly reduce the sinus tachycardia and the atrioventricular block occurring during anaphylaxis, since they are induced by histamine (33).

All of these pharmacological studies will indicate which compounds are suitable for subsequent testing on the anaphylaxis of the coronary artery in vitro and of the heart in vivo.

4. In vivo anaphylaxis in the rabbit:

This will be induced either by active sensitization with tobacco antigens or by passive transfer of rabbit anti-tobacco sera. Rabbits will be anesthetized with sodium pento-

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount	Fringe
Professional: We propose to recruit a colleague with background in immunopathology, who would have a faculty appointment	50	$12,500	$1,950
Tuition and cost of living support for a graduate student	70	8,000	
Technical			
1. Immunochemistry technician	80	10,000	1,575
2. General laboratory & animal technician	100	9,200	1,451
3. Animal caretaker	100	7,600	1,200
4. Histology technician	50	5,000	800
5. Immunopharmacology technician	40	4,000	650
Sub-Total for A		$63,926	

B. Consumable supplies (by major categories)		
1. Rabbits, special atherogenic diets for rabbits, standard diets, cage materials, approximately 6 Rhesus or African Green monkeys		12,020
2. Chemicals, biologicals, isotopes, glassware, tissue processing materials, stationary, photographic supplies, printing costs		7,000
Sub-Total for B		19,020

C. Other expenses (itemize)	
Maintenance and service contract costs on centrifuges, counting equipment, etc.	1,200
Sub-Total for C	1,200
Running Total of A + B + C	$84,146

D. Permanent equipment (itemize)	
1. Pellet maker for animal food	800
2. Mixer for animal diets	2,200
3. Two additional channels for a Grass polygraph to record blood pressure and respiration and 2 transducers for those channels	2,120
Sub-Total for D	5,120
E. Indirect costs (15% of A+B+C)	12,622
Total request	$101,888

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$69,040	$20,922	$1,200	---	$13,674	$104,836
Year 3	74,563	23,014	1,200	---	14,817	113,594

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Arteriosclerosis, glomerulonephritis and rheumatic fever*	NIH HL-01803	$97,248	December 1, 1973 to November 30, 1974
Cardiac Dysfunctions Caused by Histamine Release*	NIH 1R01 GN 20091	$38,000 per year	June 1, 1974 to May 31, 1977

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Arteriosclerosis, glomerulonephritis and rheumatic fever*	NIH HL-01803	$171,893 per year	December 1, 1974 to November 30, 1979 if approved and funded

*Research on tobacco is not proposed in any of these grants.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Carl G. Becker, M.D.

Signature Carl G. Becker Date 8/15/74

Telephone (212) 472-8954
Area Code Number Extension

Checks payable to

Cornell University Medical College

Mailing address for checks

1300 York Avenue

New York, New York 10021

Responsible officer of institution

Typed Name Thomas H. Meikle, M.D.

Title Associate Dean

Signature Thomas H. Meikle Date 8/15/74

Telephone (212) 472-5670
Area Code Number Extension

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Details of experimental design (cont'd)

barbital (30 mg/kg i.p.) and prepared for recording of the electrocardiogram, heart rate, blood pressure and bronchomotility. Antigen will be sprayed in the tracheal cannula and the parameters of cardiovascular and respiratory functions will be monitored continuously. Artificial ventilation will be provided in order to eliminate possible electrocardiographic changes due to respiratory anoxia. Cardiac autonomic interferences will be corrected pharmacologically with anticholinergics and cardioselective β-adrenergic blockers.

The involvement of the heart in hypersensitivity reactions has long been overlooked. Anaphylactic death cannot always be explained in terms of asphyxia and it is likely that cardiovascular collapse is an important factor in determining the fatal outcome. In addition, a major but not yet fully appreciated risk is that in non-fatal cases, cardiac dysfunctions may lead to irreversible cerebral and myocardial damage.

The important gain from this work, beyond examination of tobacco mediated hypersensitivity reactions of the heart, is an understanding of the seriousness of the cardiac responses to histamine, released at any site by any means. From these studies, pharmacologic means of antagonizing the serious cardiac effects of this substance will be sought.

Therefore, the results of this study should be applicable to tobacco allergy, to all antibody mediated hypersensitivities, such as allergies to pollens and inhalants, biological products and foods, and also to the effects of all histamine releasing drugs. It is surprising how little attention has been given to cardiac involvement, both directly and indirectly, in hypersensitivity phenomena.

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Details of experimental design (cont'd)

References

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6. Harkavy J: Cardiac manifestations due to allergy. NY State J of Med 64:2289, 1964.

7. Harkavy J: Cardiovascular manifestations due to hypersensitivity. NY State of Med 69:2757, 1969.

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14. Minick CR, Murphy GE and Campbell WG Jr: Experimental induction of athero-arteriosclerosis by the synergy of allergic injury to arteries and lipid rich diet. I. Effect of repeated injections of horse serum in rabbits fed a dietary cholesterol supplement. J Exp Med 124:635, 1966.

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10. Space and facilities available:

1. Laboratory space

Four large laboratories with hoods for analytical chemistry, lipid chemistry, histochemistry, and processing tissues for electron microscopy.
One small laboratory for bacteriology.
One small laboratory for immunochemistry and protein chemistry.
One small laboratory for ultramicrotomy for electron microscopy.
One small utility room for inoculations, bleeding of rabbits, preparation of special diets, and autopsy of animals.
One utility room with autoclave, automatic glassware washer, drying oven, and distillation apparatus.
Two 4° C cold rooms for protein chemistry and storage of special diets.
One room for tissue culture. This room is equipped with a small cubicle for sterile transfer, a CO_2 incubator, and an inverted microscope.

2. Offices for each investigator and a technician/secretary.

3. Animal quarters

One large animal room with 40 breeding cages and 150 other cages for maintenance of specially bred stock of rabbits and other experiments.
One small room for isolation and maintenance of approximately 50 rabbits receiving streptococcal infections.
In addition, this room has 15 metabolism cages for collection of urine from infected rabbits.
One small animal room with cages for 80 rabbits fed lipid-rich diets.
One small animal room with cages for approximately 40-50 rabbits with foreign serum induced hypertension. In addition, this room contains 9 metabolism cages for collection of urine from hypertensive and non-hypertensive rabbits. (It also contains cages for approximately 10 guinea pigs for immunization.)
One small room for storage of food, shavings, and cleaning equipment for the animal rooms.
One small room with cage cleaning facilities.
Three refrigerators - 2 for storage of dead animals and 1 for storage of special diets.

4. One Siemens Elmiskop I and one RCA EMU 3-B electron microscope with two dark rooms, one for developing plates and one for printing (departmental facility shared with other investigators).

5. A scanning electron microscope, ETEC Ultrascan, has recently been acquired as an institutional instrument and is readily available for the use of Dr. Minick and Dr. Insull one day per week. In addition, there is considerable evening and weekend time available. The facility includes a dark room, a critical point dryer, vacuum evaporator and stereoviewer. A cryostage and micromanipulator are to be delivered.

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Facilities available (cont'd)

6. Facilities for ultramicrotomy, including one Porter Blum MT-1 ultramicrotome, one LKB Ultrotome with special cutting table, one LKB knife maker, one dissecting microscope, and an oven for polymerization of embedding materials.

7. Histology laboratory facilities, including 2 American Optical microtomes, one knife sharpener, staining equipment, and paraffin oven. A Technicon is shared with the departmental histology laboratory.

8. One Beckman DB spectrophotometer equipped with automatic wavelength programmer and potentiometric recorder and 1 Coleman junior spectrophotometer.

9. One refrigerated high speed centrifuge, Beckman LB75 preparative ultracentrifuge, and 3 table centrifuges.

10. Electrophoresis equipment. Buchler apparatus for agar gel immunoelectrophoresis, analytical polyacrylamide electrophoresis and preparative polyacrylamide electrophoresis. Equipment for starch gel, starch block and agarose block electrophoresis.

11. Equipment for column chromatography including 2 fraction collectors (one fraction collector is very worn and must be replaced).

12. One bacteriology incubator, 3 water baths, and 1 constant temperature bath. Two ovens for drying and evaporation.

13. One Braun disintegrator for grinding bacteria.

14. Two distilling apparatuses, one for distillation of lipid solvents and one for double distilling water.

15. Three deep freezes and four refrigerators.

16. One vacuum-freeze lyophilizer.

17. One IEC cryostat. (The latter is departmental equipment shared with others.)

18. One analytical balance, three torsion balances, one beam balance and two scales for weighing rabbits and feed. The torsion balance in the animal room is over 10 years old and badly worn and must be replaced.

19. One Grass model 5P1 polygraph with four channels. One strain gauge pressure transducer. (This equipment is on loan from another department, must be returned, and a replacement purchased.)

20. Two pH meters.

21. Four microscopes and 2 photomicroscopes (both adapted for fluorescence microscopy).

22. Photographic apparatus for photographing gross pathology specimens.

23. One Nuclear Chicago Gamma Radiation Counter, 1185 Series

24. One Nuclear Chicago Liquid Scintillation Counter, Isocap 300

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11. Additional facilities required:

None

12. Biographical sketches of investigators:

Curricula vitae of the four investigators follows.

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13. Pertinent publications:

Reprints of the 15 publications listed below that are pertinent to this application have been supplied earlier (July 15, 1974) in triplicate to the offices of The Council for Tobacco Research.

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1. Minick CR, Murphy GE and Campbell WG Jr: Experimental Induction of Athero-arteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. I. Effect of repeated injections of horse serum in rabbits fed a dietary cholesterol supplement. J Exp Med, 124, no 4, 635-652, 1966.

2. Minick CR and Murphy GE: Experimental Induction of Atheroarteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. II. Effect of repeatedly injected foreign protein in rabbits fed a lipid-rich, cholesterol-poor diet. Am J Path, 73, no 2, 265-300, 1973.

3. Hardin NJ, Minick CR and Murphy GE: Experimental Induction of Atheroarteriosclerosis by the Synergy of Allergic Injury to Arteries and Lipid-Rich Diet. III. The role of earlier acquired fibromuscular intimal thickening in the pathogenesis of later developing atherosclerosis. Am J Path, 73, no 2, 301-326, 1973.

4. Becker CG and Murphy GE: Demonstration of Contractile Protein in Endothelium and Cells of the Heart Valves, Endocardium, Intima, Arteriosclerotic Plaques, and Aschoff Bodies of Rheumatic Heart Disease. Am J Path, 55, no 1, 1-37, 1969.

5. Becker CG and Nachman RL: Contractile Proteins of Endothelial Cells, Patelets and Smooth Muscle. Am J Path, 71, no 1, 1-22, 1973.

6. Becker CG: Demonstration of Actomyosin in Mesangial Cells of the Renal Glomerulus. Am J Path, 66, no 1, 97-110, 1972.

7. Jaffe EA, Nachman RL, Becker CG and Minick CR: Culture of Human Endothelial Cells Derived from Umbilical Veins. Identification of morphologic and immunologic criteria. J Clin Invest, 52, no 11, 2745-2756, 1973.

8. Levi R: Hypersensitivity Reactions of the Heart: An Experimental Model. Bul NY Acad Med, 46, no 11, 997, 1970.

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Pertinent publications (cont'd)

9. Levi R: Effects of Exogenous and Immunologically Released Histamine on the Isolated Heart: A Quantitative Comparison. J Pharm and Exp Therapeut, 182, no 2, 227-238, 1972.

10. Capurro N and Levi R: Anaphylaxis in the Guinea-Pig Isolated Heart: Selective inhibition by burimamide of the positive inotropic and chronotropic effects of released histamine. Brit J Pharm, 48, no 4, 620-628, 1973.

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12. Capurro N and Levi R: The Heart as a Target Organ in Systemic Allergic Reactions: Comparison of Cardiac Anaphylaxis in vivo and in vitro. Submitted for publication to Circulation Research, July 1974.

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14. Harkavy J: Cardiac Manifestations Due to Allergy. NY State J of Med, 64, no 18, 2291-2302, 1964.

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 27, 1974

Grant application No. 990

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Jacobson and Liebow

Subject: George M. Hass, M.D., Rush-Presbyterian-St. Luke's Medical Center
New application No. 990
"Pathogenesis of Cholesterol-Vitamin D-Nicotine Induced Arteriosclerosis"

History

As item No. 7 of the enclosed application states, these studies were supported by the Committee for Research on Tobacco and Health, AMA-ERF, now discontinued.

Request

Application No. 990 requests $38,099 plus 2 additional years.

Documents submitted

Enclosed is application dated April 20, 1974.

Reprints of the five papers listed at the end of Dr. Hass's CV were provided and will be forwarded to you on request.

Also provided was a manuscript (available to you if you wish) "Prevention of Cholesterol-Vitamin D-Nicotine Atheroarteriosclerosis with Thrombosis in Rabbits by Adrenalectomy."

FWN:gh
Enclosures

F.W.N.

FWN

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUN 25 1974

Application for Research Grant
(Use extra pages as needed)

Date Apr. 20,1974

1. Principal Investigator (give title and degrees): George M. Hass, M.D., Acting Chairman, Department of Pathology

2. Institution & address: Rush-Presbyterian-St. Luke's Medical Center
1753 W. Congress Parkway
Chicago, Illinois 60612

3. Department(s) where research will be done or collaboration provided: Department of Pathology

4. Short title of study: Pathogenesis of Cholesterol-Vitamin D-Nicotine Induced Arteriosclerosis

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims: The first purpose is to further establish relations between arterial disease induced by cholesterol-vitamin D-nicotine regimens and the following factors: (1) adrenocortical function, (2) hepatocellular function and (3) catecholamine function.

The second purpose is to determine whether the vasculotoxic action of nicotine in combination with modest amounts of dietary cholesterol and vitamin D can be minimized by elimination or blockade of the pathway by which enhancement of the vasculotoxic action occurs.

These aims are based principally on our past and current observations. Some have been published in detail (see Paragraph 13). Others are in manuscript form (see attached manuscript). Still others are under continuing study.

The most relevant observations have been obtained during the period of support by the Committee for Research on Tobacco and Health, AMA Research and Education Foundation. The funding by this Committee was terminated as of 02-01-74, though we have been allowed to use a small unexpended sum during the period, 02-01-74 to 02-01-75.

The content of our last Progress Report to the above Committee is as follows and summarizes the reasons for the current direction of research and our present proposal.

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Progress Report

In this report the progress since the beginning of this study will be summarized and brought up to 10-25-72, the date of this writing.

First: Chronic sublethal injections of nicotine over a period of several months in more than 100 rabbits did not produce any arterial or other disease, recognizable microscopically.

Second: In the aforementioned series of animals, nicotine had no sustained action on the levels of serum cholesterol.

Third: When daily injections of nicotine in mineral oil were given subcutaneously to rabbits maintained on a specific regimen of dietary cholesterol and subcutaneous injections of vitamin D in corn oil, a severe arterial disease developed. The regimen in the absence of nicotine was not productive of this disease. The disease usually occurred within 2-4 months and was characterized principally by peripheral calcific arteriosclerosis, intimal proliferation, atheromatous deposition and thromboarteritis.

Fourth: A controlled study of this disease by gross and detailed microscopic methods in more than 150 rabbits led to the following conclusions. First, it was apparent that all animals did not react equally but that 30 to 75 percent of those on the complete multifactorial regimen developed the disease. Second, nicotine enhanced the calcifying action of vitamin D in some unexplained way. Third, the calcification of arterial walls stimulated proliferation of the intima to an extent about equal to the thickness of arterial media affected by calcium deposition. Fourth, the loci of intimal proliferation became sites for elective accumulation of plasma lipids, even though the cholesterol levels were much lower than those ordinarily required for significant lipid accumulation. Finally, these reactive sites, especially in peripheral arteries of the skeletal muscle, ear and duodenum frequently (about 30-50 percent) were involved by arteritis and thrombosis. There was more than a passing resemblance of this disease to peripheral calcific arteriosclerosis with thrombotic complications in man.

Fifth: Peripheral calcific thromboarteritis of the type induced by nicotine was seldom noted in animals on the cholesterol-vitamin D regimen without nicotine. This fact led to a series of studies directed toward an analysis of the means by which nicotine produced the observed arterial disease. These studies involved an inquiry into lipid metabolism, adrenal function and liver function.

Sixth: Two factors pertinent to lipid metabolism were evaluated. One factor was the level of serum cholesterol. It was soon clear that the overall chronic persistent result of nicotine was to slightly depress the level of serum cholesterol. Nicotine, however, had a much more impressive effect on the levels of plasma free fatty acids (FFA). After a long series of studies with repeated determinations of plasma FFA at monthly intervals, it was concluded that rabbits could be divided into three groups. Those that had the greatest nicotine-induced rise in plasma FFA tended to develop calcific arteriosclerosis complicated by thrombosis. Those that had a modest rise in plasma FFA following injections of nicotine tended to develop calcific arteriosclerosis alone. The few animals that

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had a negligible rise in plasma FFA tended to have no peripheral arterial disease. It would be of great interest if a similar analysis could be made by clinical investigators concerned with "risk factors" in human arteriosclerosis.

Seventh: We followed the lead obtained by plasma FFA analyses with the thought that the increased level of plasma FFA induced by nicotine might be responsible for the tendency to thrombosis. ACTH and heparin, both of which produced far greater rises of plasma FFA than nicotine, were given to a series of animals on the cholesterol-vitamin D regimen with and without nicotine. There was no effect of ACTH or heparin on the arterial disease. Hence, it was concluded that the magnitude of rise of plasma FFA induced by nicotine was merely an indicator of the probability of occurrence of nicotine-induced arteriopathy and not an essential factor in pathogenesis of the arterial disease.

Eighth: Attention was next directed to the role of the adrenal gland in mediation of the action of nicotine. With the assumption that the observed effects of nicotine were adrenergic, a series of rabbits was given multiple daily injections of large amounts of adrenalin in oil for several weeks. Adrenalin did not duplicate the action of nicotine. A second series of rabbits was given reserpine in sub-lethal amounts to block the theoretical catecholamine effects of nicotine, both at the adrenal medullary and peripheral storage levels. Reserpinized animals developed severe arterial disease, much more so in the duodenal than peripheral arterial systems. This is now being restudied because of the indication that we may have struck upon a means of evaluating factors which regulate the vagaries of distribution of arteriosclerosis in man, as well as in the experimental animals.

Ninth: Inasmuch as we could not duplicate the action of nicotine by use of adrenalin or block its action by reserpine, we resorted to adrenalectomy - a difficult procedure in the rabbit. It was concluded from a series of 50 adrenal-ectomized rabbits that, if the amount of residual and regenerated adrenal tissue was more than 200 milligrams and less than about 500 milligrams, the incidence of calcific arteriosclerosis and thrombosis was greatly reduced among animals on the cholesterol-vitamin D-nicotine regimen. If the amount of residual and regenerated adrenal tissue was less than 200 milligrams (normal 1200 milligrams), the occurrence of arterial disease (1/19) was practically prevented. Current studies are directed toward evaluation of the relative importance of adrenal cortex and medulla in the mediation of the arterial disease.

Tenth: It is well known that the liver has a central role in the metabolism of catecholamines, vitamin D and substances such as nicotine. We have shown that among more than 55 rabbits surviving in good health for 6-12 months with severe progressive nodular cirrhosis, tolerance to vitamin D was reduced 50 to 100-fold. Hence, the action of nicotine was simulated by carbon tetrachloride-induced cirrhosis. However, the effects of cirrhosis and nicotine were not additive as we had reason to suspect in theory. Our present studies are directed toward further evaluation of the role of the liver in mediating the effects of nicotine and vitamin D. This is relevant to man for there is evidence that cirrhosis and cigarette-smoking seem to be synergistic as "risk factors" in arteriosclerosis.

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Eleventh: Our current and projected studies are directed toward an explanation of the undesirable effects of nicotine in the multifactorial cholesterol-vitamin D-nicotine system. There is no doubt that nicotine enhances the vasculotoxic action of vitamin D and there is more than a little evidence that excessive cigarette smoking may be associated with excess arterial calcification in man. It is also generally agreed that vitamin D or its metabolites have much to do with calcification in man. If nicotine acts as a stress factor and if its undesirable action in man is related to individual reactions to stress, our attention to the adrenal and the liver as mediators of reactivity seems justified. Reduction of adrenal function by surgical ablation minimized nicotine action but it remains for us to determine whether this was due to reduction of medullary or cortical function or both. We suspect both though we have been unable to implicate medullary function either by use of exogenous adrenalin or reserpine. To what extent an interference in liver function was involved remains a puzzle. There was a similarity between the "activation" of vitamin D, not only by nicotine but also by reduced liver function in the cirrhotic animal. It would seem advisable, therefore, for us to give increasing attention to nicotine enhancement of arterial calcification and the extent to which the regulation of calcium metabolism by mineralocorticoids, vitamin D and catecholamines or similar neurohumoral agents is involved. Our results with adrenalectomy were adequate proof that arterial calcification and subsequent thrombosis can be minimized and prevented without undue impairment of the health of the animal. If the mechanisms implicated in this prevention can be understood, prevention of calcification of arteries in man may be realized. If this can be achieved, arteriosclerosis and its complications in man can be practically eliminated. In proceeding to this end in the projected studies, a systematic study of the reasons for the susceptibility of the cirrhotic animal to arterial calcification and the resistance of the adrenalectomized animal to the same disease is being undertaken using the nicotine-cholesterol-vitamin D regimen. In carrying out this study primary attention will be given to the detection of active metabolites of vitamin D in the plasma, the binding of these metabolites to plasma proteins and the elective binding of these metabolites to receptor sites affected by nicotine.

[illegible] cholesterol-vitamin D-nicotine regimen. If the amount of residual and regenerated [illegible]

toward evaluation of the relative importance of adrenal cortex and medulla in mediation of the arterial disease.

[illegible] it is well known that the liver has a central role in the metabolism [illegible]

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8. Brief statement of working hypothesis:

There is evidence that excessive cigarette smoking is associated with an increased incidence of severe arteriosclerosis in some people. The nature of this association is not clear but it is assumed that the sympathomimetic action of nicotine is responsible. Such an assumption might well take into account the possibility that nicotine enhances the arteriosclerotic action of genetic and other "risk factors," which govern the pathogenesis of the disease. Inasmuch as we now have some experimental proof for this, as summarized in the Progress Report in paragraph 7, it becomes desirable, first to define the metabolic pathway of enhancement and then to seek [illegible] means to eliminate it.

[illegible] whether this was due to reduction of medullary or cortical function or both. We suspect both though we have been unable to implicate medullary function either by use of exogenous adrenalin or reserpine. To what extent an [illegible]

[illegible] extent to which the regulation of calcium metabolism [illegible]

9. Details of experimental design and procedures (append extra pages as necessary) i.

a. Our experimental approach depends principally on use of an animal model system which has disclosed a pathogenetic link between use of nicotine and development of severe calcific atheroarteriosclerosis complicated by thrombosis in rabbits given modest amounts of vitamin D and dietary cholesterol.

The basic regimen is as follows. Male New Zealand random-bred albino rabbits, about 4-6 months old and weighing 6-8 pounds will be used. They will be fed a diet containing sufficient cholesterol and corn oil to maintain the average serum cholesterol levels between 300 and 400 milligrams percent, at which concentration in the absence of other vasculotoxic factors, no significant atherosclerosis develops. The second "risk factor" of the regimen will be subcutaneous injection of vitamin D (Ergocalciferol) in corn oil (100,000 IU per ml) in a dosage of 25,000 IU to 100,000 IU twice every fourth week. The third "risk factor" will be nicotine, as the base (100 mgm/ml in mineral oil), given intramuscularly five days each week. The initial dose will be 20 milligrams and this will be increased 2 milligrams each week up to a maximum tolerable dose, usually less than 50 milligrams. Each experiment will vary in duration but most of them, with good fortune, will last for an average of 20-30 weeks. At the end of each experiment, complete autopsies will be done and a thorough microscopic study made of the affected organs and arterial systems (40-60 paraffin sections stained with hematoxylin and eosin). Special stains will be used when indicated.

The principal routine analytical studies will be serum cholesterol, serum calcium, and serum phosphate determinations every two months, or more often when indicated.

b. The variable of hyperlipemic hypercholesteremia

We do not intend to concentrate on this variable except insofar as dietary regulation will be used to maintain the serum cholesterol levels in the usual high adult human range where it seems to be most significant as a serious "risk factor." This may not always be possible because of the variable effects of different regimens on the health and eating habits of the animals but the average levels will not exceed 350 milligrams percent.

c. The variable of plasma FFA

Our previous studies have shown that animals with the greatest nicotine-induced rise of plasma FFA were most likely to develop severe calcific arterio-sclerotic thromboarteritis. We do not plan to explore this "genetic matter" further because ACTH or heparin, both of which induce far greater increases in plasma FFA than nicotine, did not influence the development of atheroarteriosclerosis or thrombosis. The "genetic matter" involves something more than the simple increase in plasma FFA.

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(see continuation sheet)

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

9. (continuation)

The effect of adrenalectomy on the nicotine-induced rise of plasma FFA may be studied later, if time permits. Such quantitative study requires many repeated successive spaced analyses and many controls which do not seem as relevant at this time as the proposed experiments.

d. The variable of vitamin D

It seems clear from results of our studies, to date, that the vasculotoxic effects of nicotine are mediated principally through the action of vitamin D. Nicotine enhances the calcifying action of vitamin D and in some obscure way induces a thromboarteritis in certain distal peripheral arterial systems. To some extent this also seems to be an effect of nicotine in some people. The statistical evidence indicates that heavy cigarette smoking seems often to be associated with excessive calcification of coronary arteries. It is also well known that the only useful treatment of thromboangiitis obliterans in man is to stop smoking. In our projected experiments we will use ranges of dosage of vitamin D that best bring out the vasculotoxic synergism of the nicotine-vitamin D-cholesterol regimen. For study of nicotine enhancement of the calcifying arteriosclerotic action of vitamin D, the dosage of 25,000 to 50,000 IU of vitamin D twice every fourth week is most useful. For study of enhancement of action of nicotine in production of thromboarteritis, a dosage of 50,000 to 100,000 IU twice every fourth week is the best. It is likely, judging from experience thus far, that the optimal dosage for production of vasculotoxic effects in cirrhotic animals may be no more than 1,000 IU while that in totally adrenalectomized animals may exceed 1,000,000 IU. In any event, the quantitation of relations between "risk factors" and impairment of adrenal or hepatic function will be essential if we are to draw adequate conclusions.

e. The variable of nicotine

In our first studies several years ago, nicotine was added to the diet. This proved to be unsatisfactory because of individual dietary habits. Later, nicotine was given subcutaneously in several rapidly absorbed vehicles. This led to erratic results. Finally, nicotine was dissolved in mineral oil (White Oil #35 USP, American Oil Co.) and given intramuscularly. This provided the desired degree of slow absorption and reduced the frequency of grand mal seizures. These seizures ordinarily occurred immediately after injection and were more common as the dosage was increased. However, some animals tended to react repeatedly at low dosages while others failed to react at all despite use of very high dosages. Our results indicate that the optimal dosage for obtaining the vasculotoxic effects among animals on the cholesterol-vitamin D-nicotine regimen is about 20-30 milligrams per day, though statistically significant effects have been obtained at dosages as little as 2-5 milligrams of nicotine given intramuscularly in mineral oil, no more than twice each week. In our projected studies, we plan to use the maximum effective dosages within the limits of the tolerance of the animals. Whether the limit of tolerance, as manifest by grand mal seizures, is related to the limit of tolerance to vasculotoxic effects is to be evaluated. It seems likely that this dosage may be very high in adrenalectomized animals and much lower in animals with cirrhosis or animals given reserpine.

f. The variable of adrenocortical function

It is at the level of this variable where some insight may be gained as to the pathway of mediation of the vasculotoxic action of the cholesterol-vitamin D-nicotine regimen. Analysis of the role of adrenocortical function would be simplified if there was independence of adrenal cortical and adrenal medullary activity. Unfortunately, this is not so because recent evidence strongly supports the view, long held by students of Addison's Disease, that adrenal medullary enzymatic conversion of norepinephrine to epinephrine is regulated by adrenal glucocorticoids. It has been shown that in the absence of the hypophysis, this regulation can be taken over by administration of glucocorticoids or ACTH.

It is technically difficult, if not impossible, to demedullate the adrenals

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of the rabbit without serious impairment of cortical tissue. It is possible, however, to do a complete adrenalectomy and thereby eliminate adrenal medullary tissue, unless recognized "accessory" medullary tissue remains. In our experience, if adrenalectomy is incomplete and corticosteroid therapy not used, there is a conspicuous regeneration of residual adrenal cortex, which could be interpreted as hyperplastic "accessory adrenals." If a complete adrenalectomy is done, the animal will not survive unless corticosteroid therapy is instituted. Under these conditions in our experience so-called "accessory" adrenal tissue is absent or insignificant except in rare instances. Complete adrenalectomy is best done as follows. The right adrenal is first removed by intracapsular enucleation and ablation with silver nitrate. One week later the left adrenal is surgically excised after ligation of its blood supply. The glucocorticoid, Dexamethasone (1.0 milligram) and the mineralo-corticoid, desoxy-corticosterone (0.5 milligrams) are then injected subcutaneously, daily, five days each week. After four weeks, the animals are placed on a diet containing 250-500 milligrams percent of cholesterol and administration of nicotine and vitamin D instituted. For the sake of completeness, appropriate control paired experiments will be done using animals with intact adrenals on such variations of the cholesterol-vitamin D-nicotine regimen as those being assessed in the adrenalectomized pair. We already have data using sham or non-adrenalectomized animals so that most studies will be made on fully adrenalectomized animals or at least on animals with little prospect of terminal adrenal tissue weighing more than 150 milligrams (about one-tenth the amount present in controls on the same regimen).

We expect, based upon current experience, that when there is less than about 150 milligrams of adrenal tissue at termination following adrenalectomy (usually 15-35 weeks), the regimen should have no significant vasculotoxic or thromboarteritic action. If more than about 250-350 milligrams is present at termination, some degree of vasculotoxic calcific but little or no thromboarteritis action should persist. In both instances the amount of microscopically demonstrable residual medulla will be negligible and the maintenance of good health will depend to a large extent on adequate corticosteroid therapy. The principal question to be answered, therefore, is how a small demedullated remnant of adrenal cortex supports the vasculotoxic action of the cholesterol-vitamin D-nicotine regimen. This would seem to exclude a role for epinephrine and medullary norepinephrine. It may be assumed, in the presence of adequate steroid substitution therapy, that an intrinsic essential product of cortical cell function is not involved. An alternative assumption is that support for the vasculotoxic action is provided by an extrinsic product modified by some adrenocortical enzyme system.

g. The variable of hepatic function

Our recent studies have shown that the standard regimen developed for production of cirrhosis by use of carbon-tetrachloride in the rabbit also produces an extreme sensitivity to vitamin D. This is often so pronounced that generalized arterial calcification occurs at a dosage of vitamin D less than 1-2 percent of the amount required to produce the same disease in control animals.

This proposed program, therefore, involves a continuation of these studies of the role of impaired hepatic function. The following procedure will be used.

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Carbon tetrachloride (CP) will be dissolved in mineral oil (White Oil #35 USP, American Oil Co.) to give a ten percent solution. This will be given subcutaneously at a 3-day interval, twice each week for eight weeks and thereafter, twice every other week. The dosage will be 0.3 milliliter of the 10 percent solution (0.03 milliliter of carbon tetrachloride) per pound of body weight. At the end of the fourth week of the regimen, animals will be placed upon various permutations of the cholesterol-vitamin D-nicotine regimen. Addition of cholesterol will be 250-500 milligrams per 100 grams of diet. Dosage of vitamin D will be varied from 0 to 1580 IU twice every fourth week. Dosage of nicotine will be varied from the standard amount to zero. Appropriate control animals will be placed on the same regimen first without use of carbon tetrachloride. We shall also seek a minimally effective hepatotoxic dosage schedule so as to evaluate the action of carbon tetrachloride at dosages insufficient for production of cirrhosis.

A second method for production of cirrhosis in the rabbits needs to be developed so as to distinguish the effects of cirrhosis and impaired hepatic function from those of carbon tetrachloride which has a specific action as a lipid peroxidant. The first substance to be tried will be thioacetamide. This is a controllable hepatotoxic reagent for inducing cirrhosis in the rabbit without causing too many undesirable extrahepatic effects.

In each instance the animals on the hepatotoxic regimens will be kept for periods of 6 to 12 months during which time relations between impaired hepatic function, cirrhosis and vasculotoxicity of the cholesterol-vitamin D-nicotine regimen can be assessed.

h. The variable of the hepato-adrenal axis

We have shown that there are reasons for believing that mediation of the vasculotoxic effects of the cholesterol-vitamin D-nicotine regimen involves the hepato-adrenal axis. One reason is that total adrenalectomy practically eliminates the vasculotoxic action of the regimen, even though effective corticosteroid therapy is used to sustain the health of the animals. The second reason is that the vasculotoxicity of the regimen is greatly enhanced in animals given sufficient carbon tetrachloride to produce severe nodular cirrhosis. In many ways the effects of the cirrhotic state mimic the effects of nicotine because cirrhosis in the absence of nicotine enhances the action of vitamin D and favors occurrence of thromboarteritis. In order to inquire into the antagonism between the effects of total adrenalectomy and those of cirrhosis in mediation of the vasculotoxic action of the cholesterol-vitamin D-nicotine regimen, we propose to assess the effect of combining adrenalectomy with induced cirrhosis in the same animal. Thus far, this has been accomplished in our laboratory by institution of the carbon tetrachloride regimen in adrenalectomized animals. All animals with cirrhosis subjected to adrenalectomy have, thus far, due to most unusual complications failed to survive longer than a few days. From our viewpoint, however, a persistent examination of the respective roles of impaired adrenal and hepatic function is essential to an understanding of the pathogenesis of calcific atheroarteriosclerosis with thromboarteritis induced by the cholesterol-vitamin D-nicotine regimen.

i. The variable of catecholamine action

Nicotine is a sympathomimetic reagent and has many actions which resemble those of the catecholamines. It is known that adrenalectomy eliminates the rise of plasma FFA due to the use of nicotine. It is also known that certain effects of nicotine may be minimized or eliminated by use of reagents that block adrenergic receptors. The pharmacology of the subject remains complex, and to some extent controversial, because of differences in reaction of different species and different parts of the vascular system to different dosages of nicotine.

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We propose to approach the subject by use of reserpine. The reason for use of this drug is based upon its principal peripheral pharmacologic action. It is generally accepted that reserpine blocks the uptake of norepinephrine into adrenergic sympathetic nerve terminals and thereby exposes norepinephrine to monoamine oxidase inactivation. The overall effect is a reduction in the supply of the neurotransmitter delivered to the receptors. There are a good many theories among the explanations for reserpine action but there is no doubt that it exerts a profound depressant action in prolonged experiments with rabbits on appropriate dosage schedules.

It is our purpose, therefore, to study effects of chronic reserpinization in rabbits on the cholesterol-vitamin D-nicotine regimen. A dosage of 0.35 to 0.50 milligrams intramuscularly three times each week seems effective and practical. In current pilot experiments the lower dosage of reserpine induces an extreme sensitivity to nicotine. This is often impressive, individually characteristic and contrary to expectation although pharmacologists can most likely give an adequate explanation. Perhaps, this is a manifestation of "supersensitivity." It may be that a relation between the sensitivity to nicotine and the vasculotoxicity of the cholesterol-vitamin D-nicotine regimen may be found in the general area of catecholamine function and adrenergic receptors. Whether these observations on reserpinized animals will provide some insight into the action of nicotine on the central nervous system and the neural pathway of hepato-adrenal axis mediation of its vasculotoxic effects may not be unduly speculative.

j. Schedule of proposed experiments

The following schedule and distribution of experimental animals is proposed with each group being kept at about the indicated number.

1. 5 adrenalectomized rabbits will be placed on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for a maximum of 40 weeks.

2. 5 adrenalectomized rabbits will be placed, postoperatively, on the DOCA-Decadron-cholesterol-vitamin D (1580 IU)-nicotine (20 milligrams)-carbon tetrachloride regimen for a maximum of 40 weeks.

3. 5 rabbits will be placed on the standard carbon tetrachloride regimen for twenty weeks. Following adrenalectomy they will be maintained on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for up to 40 weeks.

4. 5 rabbits will be placed on the standard carbon tetrachloride regimen for 20 weeks. Thereafter, they will be kept on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for up to 40 weeks.

5. 5 rabbits will be placed on the standard reserpine (0.35-0.50 milligrams)-cholesterol-vitamin D (100,000 IU -nicotine (20 milligrams) regimen for up to 40 weeks.

6. 5 rabbits will be placed on hepatotoxic chronic dosage of thioacetamide administered, intramuscularly, with the intent of producing a chronic cirrhosis comparable with that induced by the carbon tetrachloride regimen.

7. 5 rabbits will be kept on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for up to 40 weeks as a control group.

8. 5 rabbits will be placed on the standard DOCA-Decadron-cholesterol-vitamin D (1580 IU)-nicotine (20 milligrams) regimen for up to 40 weeks.

9. Other required control groups have either been studied in the recent past or are currently under study together with some of the experimental groups. Though the numbers of animals are not as many as we would like, the experiments are

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9. (continuation, 5)

essentially of a pilot type to be enlarged upon or modified as data accumulate from the study of animals that do not survive more than a few weeks. It is to be remembered that vasculotoxicity of the standard regimen is usually conspicuous within 12 weeks at a total vitamin D dosage of 600,000 IU. However, the mortality rates may be such that the total number of animals on experiment at any time will seldom exceed 40. Otherwise, the expense involved in their maintenance in the Animal Resources Facility at 80 cents each per day (total estimate of $32.00 per day) becomes too great.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

One histology technical laboratory is used full-time in this study.

One special laboratory of 300 square feet is used exclusively for records, files, autopsy performance and specimen storage.

Eight special research analytical laboratories contain required instruments as follows: Beckman Amino Acid Analyzer, Perkin-Elmer Atomic Absorption Spectrophotometer, DU and DK Spectrophotometers, Spinco Ultracentrifuges, Freeze-Dry Equipment, Deep-Freeze Equipment, Dual Gas Chromatographs, RCA and Phillips EM 300 electron microscopes and appropriate analytical balances. These are available for the proposed and other relevant analytical determinations.

Animal Hospital facilities for the housing and care of as many as 60 rabbits are available.

A general diet preparation kitchen is available and in use for preparation of the cholesterol diets.

A surgical operating suite required for adrenalectomy is available two mornings each week.

11. Additional facilities required:

No additional facilities are required.

12. Biographical sketches of investigator(s) and other professional personnel (append):

(see attached sheets)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

(see attached sheets)

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R: REDACTED MATERIAL

Curriculum Vitae

George Marvin Hass, M.D.

Title: Professor of Pathology, Chairman, Department of Pathology

Birthdate: REDACTED

Place of Birth:

Education: University of Iowa (B.S., 1927), Harvard University (M.D., 1929)

Honors: Junior Fellow, Society of Fellows, Harvard Univ.

Major Research Interest: Experimental and human pathology, arteriosclerosis, growth, regeneration, neoplasia

Research Support: Human and Experimental Atheroarteriosclerosis, USPHS Grant HE-03215, #35,300 (1971-72), $35,300 (1972-73);
Pathologic Effects of Lead, USPHS Grant NB-04872, $20,600 (1971-72), $20,600 (1972-73);
2-AAF Induction of Urinary Bladder Tumors, USPHS Grant CA-08857, $21,460 (1971-72), $21,460 (1972-73);
Lymphoma and Leukemia, USPHS Grant CA-11437, $24,700 (1972-73);
Miscellaneous private and institutional (Other research), $65,000 (1972-73)

Research and/or Professional Experience: Chairman, Department of Pathology, Presbyterian-St. Luke's Hospital, 1946-; Professor of Pathology, Rush Medical College, 1971-; Professor of Pathology, University of Illinois College of Medicine, 1946-1971; Director, USPHS Training Program in Experimental Pathology (NHI, 1949-1957; NIGMS, 1957-1968) (1968-1971); Consultant, Surgeon General, USPHS, 1949-1962; Chief, Dept. of Pathology, USAF School of Aviation Medicine, 1943-1946; Chief Consultant in Pathology, Surgeon General, USAF, 1946-1949; Chief Pathologist, USAF, MC, AUS, 1942-1946; Asst. Professor of Pathology, Cornell Univ., 1939-1942; Instructor to Associate in Pathology, Harvard Univ., 1932-1939; Junior Fellow, Society of Fellows, Harvard Univ., 1934-1937; Resident in Pathology, Peter Bent Brigham Hospital, Boston, 1932-1934; Resident in Pathology, Children's Hospital, Boston, 1931-1932; Interne in Pathology, Peter Bent Brigham Hospital, Boston, 1930-1931; Research Fellow, Physiology, Harvard University, 1929-1930

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Selected Relevant References

1. Hass, George M., Trueheart, Richard E. and Hemmens, Anne
Experimental atheroarteriosclerosis due to calcific medial degeneration and hypercholesteremia.
Am. J. Path., 38:289-323, 1961

2. Hass, George M.
Metabolic and Nutritional Factors in Peripheral Vascular Disease.
Chapter 8. In: The Peripheral Blood Vessels. International Academy of Pathology, Monograph No. 4, The Williams and Wilkins Company, 1963, pp. 157-204

3. Hass, George M., Landerholm, Wayne and Hemmens, Anne
Production of calcific atheroarteriosclerosis and thromboarteritis with nicotine, vitamin D and dietary cholesterol.
Am. J. Path., 49:739-771, 1966

4. Hass, George M., Henson, Donald E., Scott, Richard A., McClain, Eldon C. and Hemmens, Anne
Influence of cirrhosis on production of atheroarteriosclerosis and thromboarteritis with vitamin D and dietary cholesterol.
Am. J. Path., 57:405-429, 1969

5. Scott, Richard A., Henson, Donald E., Lesak, Anne, Turner, Robert J., Malikova, Stanislava and Hass, George M.
Relations between metabolic increase of plasma free fatty acids and the occurrence of arteriosclerotic thromboarteritis in rabbits.
Am. J. Path., 70:209-233, 1973

Chief Pathologist, USAF, MC, AUS, 1942-1946; Asst.

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R: REDACTED MATERIAL

Curriculum Vitae

Reuben Eisenstein, M.D.

Title:	Attending Pathologist, Presbyterian-St. Luke's Hospital and Professor of Pathology, Rush Medical College
Place of Birth:	REDACTED
Birthdate:	REDACTED
Education:	Tulane University (B.S., 1949); Louisiana State University (M.D., 1953)
Honors:	Alpha Omega Alpha
Major Research Interest:	Connective Tissue Disorders
Research Support:	USPHS grant HL-14609, 1972-1975 ($29,849 annually), "The Organization of Arterial Ground Substance." Chicago Heart Association ($10,450 annually, 1973-1974), "A Proteinase Inhibitor in Vascular Walls."
Research and/or Professional Experience:	Professor, Pathology, Rush Medical College, 1971-; Professor, Pathology, University of Illinois, 1969-1971; Assoc. Prof. Pathology, Univ. of Illinois, 1965-1969; Asst. Prof. Pathology, Univ. of Illinois, 1960-1965; Assoc. Att. Pathologist, Presbyterian-St. Luke's Hospital, 1963-; Asst. Attending Pathologist, Presbyterian-St. Luke's Hospital, 1960-1963; Resident, Pathology, Presbyterian-St. Luke's Hospital, 1957-1960; Resident, Pathology, Mt. Sinai Hospital, 1954-1955; Interne, Mt. Sinai Hospital, New York, 1953-1954. Member, Cardiovascular B Study Section, NIH, 1967-1971

10035442 48

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Recent Relevant Publications

1. Eisenstein, R., Ellis, H. and Rosato, J.
In vitro studies of vitamin D induced aortic calcification.
Proc. Soc. Exp. Biol. and Med., 132:58, 1969

2. Eisenstein, R., Scott, R.A. and Lesak, A.
Altered lipid binding by mineralized aortic elastin.
Arch. Path., 92:301-306, 1971

3. Eisenstein, R.
Pathological Calcification.
In: The Biochemistry and Physiology of Bone, ed. G.H.Bourne, Academic Press, 1972, Vol. 2, p. 357

4. Eisenstein, R., Arsenis, C., Sorgente, N. and Kuettner, K.E.
Effect of vitamin D on serum and tissue lysozyme.
AMA Arch. Path., 92:301, 1971

5. Eisenstein, R., Larsson, S., Sorgente, N. and Kuettner, K.E.
Collagen-proteoglycan relationships in epiphyseal cartilage.
Am. J. Path., 73:443-452, 1973

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R: REDACTED MATERIAL

Curriculum Vitae

Robert E. Lee, Jr., M.D., Ph.D.

Title: Assistant Attending Pathologist, Presbyterian-St. Luke's Hospital, Assistant Professor of Pathology, Rush Medical College

Birthdate: REDACTED

Place of Birth:

Education: St. Mary's College, Winona, Minn. (B.S., 1954); Loyola Univ. Graduate School (M.S., Anatomy, 1958), Loyola Univ. Stritch School of Med. (Ph.D., Anatomy, 1962); Stritch School of Med., (M.D., 1965)

Research Support: Schweppe Fellowship, $10,000 (1973-74)

Research and/or Professional Experience: Assistant Attending Pathologist, Presbyterian-St. Luke's Hospital and Assistant Professor of Pathology, Rush Medical College, 1972-; Attending Pathologist, Walter Reed General Hospital, 1970-1972; Schweppe Foundation Fellow, Presbyterian-St. Luke's Hospital, 1969-1970 and 1973-1975; Resident, Pathology, Presbyterian-St. Luke's Hospital, 1966-1970; Clinical Fellow, Pathology, American Cancer Society, Presbyterian-St. Luke's Hospital, 1967-1969; Intern, Pathology, Presbyterian-St. Luke's Hospital, 1965-1966; Research Fellow, Loyola Univ., 1962-1965; Royal E. Cabell Fellow, Loyola Univ., 1960-1961; Instr. Pathology, Univ. of Illinois, 1968-1970; Assistant, Pathology, Univ. of Illinois, 1966-1968; Assistant Instructor, Cook County Hosp. School of Nursing, 1958-1959; Laboratory Teaching Asst., Loyola University, 1956-1958 and 1959-1962

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Recent Relevant Publications

Robert E. Lee, Jr., M.D., Ph.D.

1. Lee, R.E., Jr., Hobart, E. and Aras, A.
   Inhibition of ATP-induced contraction of human myofibrils in vitro by antihuman myofibril rabbit serum. Lab. Invest., 22:504, 1970

2. Lee, R.E., Jr., Hobart, E., Aras, A. and Andresen, R.
   Electron microscopy of human myofibrils resistant to ATP-induced contraction following reaction with antiserum.
   Am. J. Path., 62:43a, 1971

3. Hobart, E.D., Lee, R.E., Jarosz, H. and Aras, A.
   A severe myopathy produced in guinea pigs with a diet containing lead and deficient in vitamin C.
   Fed. Proc., 30:522, 1971

4. Lee, R.E., Jr., Hughes, F.W., Aras, A. and Hass, G.M.
   Effects of extraction of myosin or actin on weight contraction and ultrastructure of myofibrils reacted with antimyofibril serum.
   Lab. Invest., 28:405-406, 1973

5. Hughes, W.F., Lee, R.E., Jr., Olson, R.H. and Hobart, E.D.
   Light and electron microscopic studies of a myopathy produced in guinea pigs fed a diet containing lead and deficient in vitamin C.
   Am. J. Path., 70:62a-63a, 1973

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
George M. Hass, M.D.		REDACTED
Reuben Eisenstein, M.D.		
Robert E. Lee, Jr., M.D.		
Technical		REDACTED
B. Zidonis (Histo. Tech.)		
M. Kedys (Res. Tech.)		
J. Dikinis (Chem. Tech.)		
R. Turner (Chief Tech.)		

Sub-Total for A: 17500

B. Consumable supplies (by major categories)

Cost of 100 rabbits	750
Cost of diet cholesterol	500
Cost of laboratory reagents	1500
Cost of glassware, etc.	1200

Sub-Total for B: 3950

C. Other expenses (itemize)

Animal Hospital care of a colony of 40 rabbits, each at 80 cents per day

Sub-Total for C: 11680

Running Total of A + B + C: 33130

D. Permanent equipment (itemize)

none

Sub-Total for D: none

E. Indirect costs (15% of A+B+C): 4969

Total request: 38099

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	18550	4345	12380	none	5291	40566
Year 3	19663	4779	13123	none	5635	43200

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Human and Experimental Atheroarteriosclerosis	USPHS HE-03215	$70,600	1971-1973
Miscellaneous Institutional		65,000	1972-1973, 1974-1975
Pathogenesis of Nicotine Induction of Calcific Athero-arteriosclerosis with Thromboarteritis in Rabbits	AMA Comm. Res. on Tobacco and Health	142,080	1967 - 1975
(see continuation sheet)			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Institutional		$65,000	1975-

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name George M. Hass, M.D.

Signature George M. Hass Date 06/03/74

Telephone 312 (Area Code) 942 (Number) 5219 (Extension)

Checks payable to

Rush Presbyterian-St. Luke's Med. Ctr.

Mailing address for checks

1753 W. Congress Parkway

Chicago, Illinois 60612

Responsible officer of institution

Typed Name William Hejna, M.D.

Title Vice Pres., Med. Affairs and Dean, Rush Medical College

Signature William Hejna

Telephone 312 (Area Code) 942 (Number) 5477 (Extension)

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16. (Continuation)

Currently Active

The Organization of Arterial Ground Substance	USPHS HL-14609	$89,547	1972-1975
A Proteinase Inhibitor in Vascular Walls	Chicago Ht. Assoc.	10,450	1973-1974
Schweppe Fellowship	Schweppe Fdn.	10,000	1973-1974
Institutional		7,500	1973-1974

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RUSH-PRESBYTERIAN-ST. LUKE'S MEDICAL CENTER

1753 West Congress Parkway, Chicago, Illinois 60612

COMMITTEE ON HUMAN INVESTIGATION

SUBJECT: Clinical Research and Investigation NOT Involving Human Beings.

Date: June 3, 1974

PRINCIPAL INVESTIGATOR: George M. Hass, M.D.

TITLE OF PROJECT: Pathogenesis of Cholesterol-Vitamin D-Nicotine Induced Arteriosclerosis.

SOURCE OF FUNDS: Council for Tobacco Research - U.S.A., Inc.
(Grant No. if applicable)

PROJECT PERIOD: January 1, 1975 - January 1, 1978

We hereby certify that this proposal does not involve clinical investigation using human subjects or the administration of personality tests, inventories, questionnaires or other experimental procedures to human subjects below the college level.

Signature: George Hass MD
Principal Investigator

Signature: n/a
Section Head (if applicable)

Signature: George Hass MD
Department Chairman

Signature: William Hejna
Institutional Official

1003544255

PLEASE SUBMIT TO THE COMMITTEE ON HUMAN INVESTIGATION.

COPY OF PROPOSAL MUST BE SUBMITTED.

APPROVED: Paul E. Carson DATE: 6-10-74

Paul E. Carson, M.D.
Chairman
Committee on Human Investigation

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

#843A HERD

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 31, 1974

Grant application No. 843A

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Feldman, and Sommers (informational copy for Dr. Meier)

Subject: J. Alan Herd, M.D. (formerly A. Clifford Barger, M.D.)
Harvard Medical School, Boston
Continuation application No. 843A
"Behavioral Hypertension and Arteriosclerosis in the Squirrel Monkey: Effects of Nicotine and Carbon Monoxide".

History

The preceding grant, entitled "Behavioral Hypertension and Arteriosclerosis: Effects of Nicotine and Carbon Monoxide" under the direction of Dr. A. Clifford Barger, is now in the final year of a three year program, with a level of support of $50,000 per annum.

Request

Application No. 843A requests $50,966 plus two additional years. As this request is for "continuation", it competes on the same basis as a new application.

Documents Submitted (attached)

1. Letter from Dr. Herd dated July 25, 1974.

2. Application dated July 17, 1974.

Comment

As suggested by Dr. Hockett, we enclose a copy of "The Physiology of Strong Emotions" by J. A. Herd: the 16th Annual Bowditch Lecture, 1971.

F.W.N.

FWN:lb
Encs.

1003542257

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HARVARD UNIVERSITY

Department of Psychiatry
(617) 734-3300

LABORATORY OF PSYCHOBIOLOGY
HARVARD MEDICAL SCHOOL
25 SHATTUCK STREET
BOSTON, MASSACHUSETTS 02115

July 25, 1974

Robert C. Hockett, Ph.D.
Research Director
The Council for Tobacco Research-U.S.A.
633 Third Avenue
New York, N.Y. 10017

Re: CTR 843

Dear Dr. Hockett:

Enclosed is a grant application entitled "Behavioral hypertension and arteriosclerosis in the squirrel monkey: effects of nicotine and carbon monoxide." This is a request for research support of a program that is an extension of that currently supported by the Council. Because of our discussions with you and Dr. Lisanti during our Site Visit on May 29, 1974, we have decided I should be designated as Principal Investigator with Dr. Barger and Dr. Dews designated as Co-principal Investigators.

This application contains a proposal to continue the study of behavioral hypertension in the squirrel monkey that we reported to the Site Visit team on May 29th. It also contains a proposal for a study of arterial wall permeability to be carried out in squirrel monkeys in the presence and absence of systemic arterial hypertension, with and without concurrent administration of nicotine and carbon monoxide. Preliminary experiments with rats have demonstrated the feasibility of this project and a description of preliminary results will be included in our Progress Report for 1974.

We are grateful for the time you and your Site Visit team spent with us in discussion of our research program. I hope we can continue to work together in our studies of hypertension and arteriosclerosis in non-human primates.

Sincerely,

J. A. Herd, M.D.
Associate Professor
of Psychobiology

JAH/qr

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

#8454

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

RECEIVED
JUL 29 1974

Application for Research Grant
(Use extra pages as needed)

Date: July 17, 1974

1. Principal Investigator (give title and degrees):
J. Alan Herd, M.D., Assoc. Professor of Psychobiology, Department of Psychiatry
Peter B. Dews, M.B., Ch.B., Ph.D., Stanley Cobb Professor of Psychiatry and Psychobiology
A. Clifford Barger, M.D., Robert Henry Pfeiffer Professor of Physiology

2. Institution & address:
New England Regional Primate Research Center
Laboratory of Psychobiology, Harvard Medical School
Department of Physiology, Harvard Medical School

3. Department(s) where research will be done or collaboration provided:
Department of Psychiatry
Department of Physiology

4. Short title of study:
Behavioral Hypertension and Arteriosclerosis in the Squirrel Monkey: Effects of Nicotine and Carbon Monoxide.

5. Proposed starting date: January 1, 1975.

6. Estimated time to complete: 3 years (January 1, 1975 to December 31, 1977).

7. Brief description of specific research aims:
(1) To determine the effects of blood pressure, diet, behavioral procedures, nicotine and carbon monoxide on entry, accumulation, and synthesis of cholesterol, triglycerides, and other solutes in carotid arteries, femoral arteries, coronary arteries, and the aorta in unanesthetized squirrel monkeys.

(2) To determine the effects of nicotine and carbon monoxide administered over long periods of time on blood pressure in the unanesthetized squirrel monkey during behavioral procedures.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

	% time	Amount (incl. fringe benefits)
Professional (give % time of investigator(s) even if no salary requested)		
J. A. Herd		REDACTED
P. B. Dews		REDACTED
A. C. Barger		REDACTED
		REDACTED
Technical		
S. A. Grose		REDACTED

Sub-Total for A $ 27,000.00

B. Consumable supplies (by major categories)

Animal purchase; 100 rats @ $3.00	$ 300.00
18 Squirrel monkeys @ $150.00	2,700.00
Animal care, 12 monkeys @ $1.10 per diem	4,818.00
Radioisotopes	2,000.00
Physiological supplies	1,000.00
Electrical supplies	1,000.00
Surgical supplies	500.00
Pathology	1,000.00

Sub-Total for B $ 13,318.00

C. Other expenses (itemize)

Art work, photography, publication expenses	$1,500.00
Maintenance of equipment	1,000.00
Machine shop expenses	1,000.00
Travel (domestic)	500.00

Sub-Total for C $ 4,000.00

Running Total of A + B + C $ 44,318.00

D. Permanent equipment (itemize)

None

Sub-Total for D 0

E. Indirect costs (15% of A+B+C) E $ 6,648.00

Total request $50,966.00

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$29,025.00	13,318.00	4,000.00	0	6,951.00	53,294.00
Year 3	31,202.00	13,318.00	4,000.00	0	7,278.00	55,798.00

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

8. Brief statement of working hypothesis:

The level of systemic arterial blood pressure is the major determinant of the rates at which cholesterol and triglycerides accumulate in the media of large arteries in the squirrel monkey. Nicotine and carbon monoxide administered in small amounts over long periods of time have inconsequential effects on the long-term hypertensive and atherosclerotic responses to behavioral procedures and atherogenic diets.

9. Details of experimental design and procedures (append extra pages as necessary)

(1) Entry, accumulation, and synthesis of cholesterol, triglycerides, and other solutes in the walls of large arteries.

(a) experiments performed in vivo. Preliminary experiments will be conducted with rats and definitive experiments will be performed with squirrel monkeys.

(i) general procedure. Animals will be prepared with small plastic catheters implanted in the right internal iliac artery and vein. The rates at which various solutes accumulate in the walls of large arteries will be determined by infusing radioactively-labeled solutes intravenously and measuring the radioactivity in large arteries excised after several hours of infusion. Concentrations of radioactively-labeled solutes in arterial blood will be determined by measuring the radioactivity in blood samples through arterial catheters at various times throughout each experiments. Rates of infusion will be adjusted to maintain constant arterial concentrations of radioactively-labeled solutes for 2 to 4 hours. Radioactively-labeled solutes to be infused include ^{14}C- and ^{3}H-antipyrine, ^{14}C- and ^{3}H-sucrose, ^{14}C- and ^{3}H-inulin, ^{125}I-albumin, ^{14}C-cholesterol, and ^{3}H-acetate. Arteries to be excised and analyzed for radioactivity include carotid, femoral, and coronary arteries as well as segments of thoracic and abdominal aortas.

The rates at which radioactively-labeled solutes enter the walls of large arteries will be determined by infusing ^{14}C- and ^{3}H-labeled solutes in the same subject for different periods of time and by excising paired arteries one at a time at different times. For example, ^{3}H-inulin will be infused for 4 hours and ^{14}C-inulin will be infused from the 2nd to the 4th hours. The right carotid artery will be taken out after 3 hours of infusion and the left carotid artery will be taken out after 4 hours of infusion. In this way concentrations of ^{14}C- and ^{3}H-labeled inulin will be determined following 1, 2, 3, and 4 hours of infusion. The right carotid artery will be excised after 1 hour of infusion of ^{14}C-inulin and 3 hours of infusion of ^{3}H-inulin. The left carotid artery will be excised after 2 hours of infusion of ^{14}C-inulin and 4 hours of infusion of ^{3}H-inulin.

Localization of radioactivity in walls of arterial segments will be determined using autoradiography. ^{14}C-labeled solutes will be infused intravenously and paired arteries will be excised one at a time at different times, distended by intra lumenal pressure of 100 mm Hg, frozen, cut in cross section, and clamped against photographic film. By comparing autoradiographs prepared

10035442 61

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from identical arteries after different periods of infusion, it will be possible to determine the rates at which radioactively-labeled solutes move through each layer of the arterial wall.

Entry, accumulation, and synthesis of cholesterol and triglycerides in the walls of arteries will be determined using continuous intravenous infusions of ^{3}H-acetate and ^{14}C-cholesterol. After several hours of infusion, segments of arteries will be excised, homogenized, and extracted in chloroform-methanol solution. After evaporation of the chloroform and methanol the amounts of ^{14}C and ^{3}H extracted from each arterial segments will be measured as well as the total amount of lipid extracted. Samples of arterial blood will also be extracted in chloroform-methanol solution and amounts of ^{14}C and ^{3}H measured.

(ii) short-term experiments. In these studies control and experimental observations will be made during the same period of infusion. Each arterial segment will serve as its own control by infusing solutes labeled with ^{3}H and ^{14}C for different periods of time or comparisons will be made between results obtained using segments from right and left carotid or femoral arteries. Effects of high and low blood pressure on solute accumulation will be determined by cannulating one carotid or one femoral artery at its proximal end. Pressure in the cannulated artery will be lower than in the contralateral artery and blood will be allowed to flow in retrograde fashion through the artery out the proximal end through the cannula and into an adjacent vein. This procedure will serve to establish a continuous flow of arterial blood through the segment at reduced pressure. The effects of various agents on arterial permeability will be tested by infusing a solute labeled with ^{3}H during the control period, excising one of a pair of arteries, infusing the same solute labeled with ^{14}C while administering some agent such as carbon monoxide or nicotine and excising the contralateral artery after an appropriate period of time. Agents to be tested include norepinephrine and angiotensin II as well as carbon monoxide and nicotine.

(iii) long-term experiments. In these studies, control and experimental observations will be made on one of a pair of arteries excised at different times under different conditions. The rates at which various solutes accumulate in the walls of large arteries will be determined by infusing radioactively-labeled solutes intravenously and measuring the radioactivity in one artery excised under anesthesia after several hours of infusion without anesthesia. Following excision, the animal will be allowed to recover from surgery and anesthesia. Subsequently, the effects of behavioral hypertension, long-term administration of an atherogenic diet, chronic administration of carbon monoxide in the inspired air, and daily oral administration of nicotine will be determined. Radioactively-labeled solutes will again be administered by continuous intravenous infusion, arterial blood will be sampled intermittently, and the contralateral artery will be excised after the same period of infusion as was used during the control observation. A separate group of animals will be used to determine the effects of removing one carotid artery on entry and accumulation of solutes in the contralateral artery.

(b) experiments performed in vitro. The greatest difficulty encountered in the study of arterial segments perfused in vitro is to maintain the integrity of the endothelium. Arterial segments removed from the body frequently show swelling of endothelial cells and abnormalities of their nuclei within approximately 2 hours. In all our experiments, endothelial integrity will be assessed using electron micrographs. These experiments will be carried out in collaboration with Dr. Ramzi S. Cotran, Professor of Pathology at Harvard Medical School and The Peter Bent Brigham Hospital.

(i) general procedures. In vitro experiments will be performed with segments of carotid arteries, femoral arteries, and segments from the arch of the aorta which are free of small branches. Each segment will be tied at each end to soft plastic tubing and suspended in Krebs-Ringer bicarbonate buffer

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solution with glucose added to a concentration of 10 mM. The lumen will be filled with plasma that has been equilibrated with a mixture of oxygen in the ratio of 95/5 and the same mixture of gases will be bubbled continuously through the buffer solution surrounding the excised perfused artery. The tubing attached to each end of the arterial segment will be filled with plasma, connected to a strain gauge pressure transducer, and a microliter syringe. Static and dynamic pressures will be controlled by a pressure resevoir and a reciprocating syringe pump. At the beginning of each experiment, pressure-volume relations will be determined and a pressure-volume curve constructed. During each experiment , pressure and volume measurements will be repeated periodically and the fluid in the lumen of the artery will be replaced by fresh fluid from the syringe and tubing.

Bulk flow of water will be determined from the amount of fluid that must be added to the system to maintain pressure at initial values. Rates of flow will be determined at high and low pressures and during application of static and pulsatile pressures.

Rates of diffusion for solutes through the walls of arterial segments will be determined by perfusing the arterial lumen with fluid at atmospheric pressure containing known concentrations of radioactively-labeled solutes. The rates at which solutes diffuse through the arterial wall will be determined by measuring the amounts of radioactivity in fluid surrounding the arterial segment.

At the end of each experiment each arterial segment will be examined for endothelial integrity and tested for leaks. Before removing the artery from the perfusion system a suspension of ^{85}Sr-microspheres will be perfused at high pressure and amounts of radioactivity will be measured in the fluid surrounding the arterial segment. Endothelial integrity will be determined using electron micrographs.

(ii) short-term experiments. In these studies control observations and experimental interventions will be made during the same period of perfusion. Each arterial segment will serve as its own control or comparisons will be made between results obtained using right and left carotid or femoral arteries. During control periods bulk flow of water and movement of solutes through the arterial walls will be determined. Subsequently, the effects of high and low blood pressures, the effects of steady and pulsatile pressures, the effects of mechanical abrasion of the endothelium, the effects of hyaluronidase applied to the endothelium, and the effects of norepinephrine, angiotensin II, carbon monoxide, and nicotine in the perfusion fluid on arterial permeability to water and solutes will be determined.

(iii) long-term experiments. In these studies, control observations and experimental interventions will be made during different periods of perfusion with right and left carotid or femoral arteries. Animals will be anesthetized, one of a pair of arteries will be removed for perfusion under control conditions and then the animal will be allowed to recover from surgery and anesthesia. Subsequently, the effects of behavioral hypertension, long-term administration of an atherogenic diet, chronic administration of carbon monoxide in the inspired air, and daily administration of nicotine in the drinking water on arterial permeability to water and solutes will be determined. A separate group of animals will be used to determine the effects of removing one of a pair of arteries on permeability characteristics of the contralateral artery.

(2) Behavioral hypertension in the squirrel monkey during chronic administration of carbon monoxide and nicotine. Previous studies performed under a grant from the Council for Tobacco Research-U.S.A., Inc. (Grant No. CTR 843) demonstrated that nicotine administered subcutaneously to squirrel monkeys suppressed rates of responding on a lever under certain behavioral schedules. These studies will be continued and extended to include measurements of heart rate, blood pressure, and cardiac output during chronic administration of carbon monoxide

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in the inspired air and nicotine in the drinking water.

Behavioral schedules that exert persistent control over skeletal muscle movements also exert predictable and persistent effects on the cardiovascular system. Squirrel monkeys maintained for several months in daily sessions of schedules occasionally associated with delivery of noxious stimuli eventually develop sustained arterial hypertension. The effects on blood pressure of these daily sessions are not due simply to the delivery of noxious stimuli. All of the animals in training receive the greatest number of stimuli during the initial stages but at this time arterial pressures are not elevated above normal control values. As training progresses and each animal begins to press the lever more rapidly, the number of noxious stimuli delivered gradually decreases but mean arterial blood pressure rises. Continuous measurements of blood pressure 24 hours a day reveal that elevations of arterial blood pressure induced by behavioral techniques eventually persist between experimental sessions.

(i) general procedures. All animals to be studied will be prepared with systemic arterial and right atrial catheters for measurement of arterial blood pressure, right atrial pressure, and cardiac output. After recovery from surgery, each monkey will be conditioned to respond by pressing a lever under schedules in which lever pressing is reinforced by termination of a stimulus associated with the presentation of noxious stimuli. For example, under a fixed-ratio schedule, a fixed number of responses is required for reinforcement and under a fixed-interval schedule, the first response after a fixed time interval has elapsed is reinforced. If a monkey occasionally receives noxious stimuli in the presence of a light, and lever-pressing responses turn off the light, the animal will tend to respond by pressing the lever whenever the light is on. Thus, complex patterns of responding in the presence of a light can be maintained over long periods of time even though noxious stimuli are only infrequently delivered.

(ii) short-term experiments. Both conditional and unconditional cardiovascular responses to environmental stimuli will be studied. Animals will be acclimated to primate restraining chairs and isolated in temperature controlled chambers for a period of 16 hours before experiments begin. During this time, control measurements of heart rate, blood pressure, and cardiac output will be made. The onset of each experimental session will be signalled by turning on a small panel light located on the front of the restraining chair. Noxious electrical stimuli will be delivered occasionally in the presence of the panel light and will never be delivered in the absence of the light. The intensity of the noxious electrical stimuli and the frequency of their delivery will be adjusted systematically to determine the schedule of stimuli that has the maximum effect on heart rate and blood pressure. Some animals will be trained to press a lever to turn off the panel light and some will be untrained. As soon as stable performances and reproducible changes in cardiovascular function have been established, various ambient temperatures, various concentrations of oxygen, carbon dioxide, and carbon monoxide in inspired air, and administration of small amounts of nicotine infused intravenously will be tested for their effects on heart rate, blood pressure, cardiac output, and behavioral performances. Those schedules of environmental stimuli which have the greatest effects on cardiovascular function will be selected for further study of their long-term effect on arterial blood pressure and aortic atherosclerosis.

(iii) long-term experiments. Animals trained to press a lever to turn off a small panel light occasionally associated with delivery of noxious electrical stimuli eventually develop sustained elevations of arterial blood pressure that persist between daily experimental sessions. When sustained elevations in blood pressure have been induced using behavioral techniques, each animal with behavioral hypertension will be studied under various procedures to determine which schedules promote the return of blood pressure to control values and which

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cause behavioral hypertension to persist. Some animals will be left in their home cages for several weeks, some will be studied under extinction schedules, and some will be trained on schedule previously found to be associated with normal levels of blood pressure. In some of these hypertensive animals, the effects of carbon monoxide in the inspired air and nicotine in the drinking water administered continuously for several weeks will be studied for their effects on cardiovascular function and behavioral performances.

Eventually, some of the animals will develop elevated levels of arterial blood pressure that will not return to control values under any schedule of environmental stimuli. These animals with permanent hypertension will be fed an atherogenic diet with and without administration of carbon monoxide or nicotine. Effects of this regimen on aortic atherosclerosis in the squirrel monkey with behavioral hypertension will be determined by comparisons between the severity of atherosclerotic lesions seen in hypertensive animals and those seen in animals with normal blood pressure on the same regimen.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Several thousand square feet of office, laboratory, and animal care space are available in the New England Regional Primate Research Center and the Harvard Medical School. Fully equipped sterile surgical operating rooms, electrical and mechanical workshops and a photographic dark room are available for use on this project. Equipment available includes standard physiological recording equipment, behavioral programming and recording equipment, isolation chambers, primate restraining chairs, syringe infusion pumps, a cryostat microtome, a gamma scintillation spectrometer, a beta liquid scintillation spectrometer, and a PDP-8/L computer.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

R: REDACTED MATERIAL

JAMES ALAN HERD

Born REDACTED

1956	M.D. University of British Columbia, Canada
1956-57	Intern, Vancouver General Hospital, Vancouver, British Columbia
1957-58	Assistant Resident in Medicine, Vancouver General Hospital
1958-59	Associate Resident in Pathology, Vancouver General Hospital
1959-60	Research Fellow in Medicine, Harvard Medical School Assistant in Medicine, Peter Bent Brigham Hospital Life Insurance Medical Research Fund Fellowship
1960-61	Research Fellow in Physiology, Harvard Medical School Life Insurance Medical Research Fund Fellowship
1961-62	Research Fellow in Physiology, Harvard Medical School Advanced Research Fellowship of the American Heart Assoc.
1962-63	Assistant Resident in Medicine, Peter Bent Brigham Hospital Webster-Underhill Fellowship
1963-64	Instructor in Physiology, Harvard Medical School
1963-68	Established Investigator of the American Heart Association
1964-67	Associate in Physiology, Harvard Medical School
1967-69	Assistant Professor of Physiology, Harvard Medical School
1969-73	Research Scientist Development Awardee, NIMH
1969-74	Associate Professor of Physiology, Harvard Medical School
1974-	Associate Professor of Psychobiology, Harvard Medical School

Memberships:

REDACTED

REDACTED

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R: REDACTED MATERIAL

PETER B. DEWS

Born REDACTED

1944	M.B. & Ch.B., University of Leeds, England
1951	Ph.D., University of Minnesota (in Physiology) Minneapolis
1959	M.A., Harvard University (hon.), Cambridge, Massachusetts
1944-45	House Physician, Grimsby & District General Hospital, England
1945-46	Demonstrator in Pharmacology, University of Leeds, England
1946-47	Lecturer in Pharmacology, University of Leeds, England
1948-49	Wellcome Research Fellow in Pharmacology, Wellcome Research Laboratories, Tuckahoe, New York
1950-51	Fellow and First Assistant in Physiology, Mayo Foundation, Section on Physiology
1952	Research Associate in Division of Biometry and Medical Statistics, Mayo Clinic
1953-54	Instructor in Pharmacology, Harvard Medical School
1954-56	Associate in Pharmacology, Harvard Medical School
1956-58	Assistant Professor of Pharmacology, Harvard Medical School
1959-62	Associate Professor of Pharmacology, Harvard Medical School
1962	Stanley Cobb Professor of Psychiatry & Psychobiology, Harvard Medical School

Memberships:

REDACTED

REDACTED

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R: REDACTED MATERIAL

A. CLIFFORD BARGER

Born [redacted]

U.S. Citizen

Born [redacted]

1939	A.B., Harvard University, Cambridge, Massachusetts
1943	M.D., Harvard University, Cambridge, Massachusetts
1938-41	Research Assistant (part-time), Harvard Fatigue Laboratory
1943	Medical House Officer, Peter Bent Brigham Hospital, Boston
1944-45	United States Army: Climatic Research Laboratory
1945	Assistant Resident in Medicine, Peter Bent Brigham Hospital
1946-47	Research Fellow in Physiology, Harvard Medical School
1946-53	Assistant in Medicine, Peter Bent Brigham Hospital, Boston
1948-50	Instructor in Physiology, Harvard Medical School
1950-53	Associate in Physiology, Harvard Medical School
1953	Associate Staff, Peter Bent Brigham Hospital, Boston
1953-55	Assistant Professor in Physiology, Harvard Medical School
1955-61	Associate Professor in Physiology, Harvard Medical School
1959	Consultant in Physiology (Medicine), Peter Bent Brigham Hospital, Boston
1959-60	Commonwealth Fund Fellow
1960	Consultant in Cardiovascular Physiology, World Health Organization
1961	Professor of Physiology, Harvard Medical School
1963	Robert Henry Pfeiffer Professor of Physiology, Harvard Medical School

Memberships:

[redacted]

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

13. Publications (J. Alan Herd)

(1) Herd, J.A., Goodman, H.M. and Grose, S.A.: Blood flow rates through adipose tissues of unanesthetized rats. Am. J. Physiol. 214:263-268, 1968.

(2) Herd, J.A., Morse, W.H., Kelleher, R.T. and Jones, L.G.: Arterial hypertension in the squirrel monkey during behavioral experiments. Am. J. Physiol. 217:24-29, 1969.

(3) Benson, H., Herd, J.A., Morse, W.H. and Kelleher, R.T.: Behavioral induction of arterial hypertension and its reversal. Am. J. Physiol. 217:30-34, 1969.

(4) Herd, J.A.: The physiology of strong emotions: Cannon's scientific legacy re-examined. Physiologist 15:5-16, 1972.

(5) Jones, L.G. and Herd, J.A.: Autoradiographic visualization of ^{85}Kr in the normal dog kidney. Am. J. Physiol. 226:886-892, 1974.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Specialized Center of Research in Hypertension	U.S.P.H.S. (HL 14150)	1,709,167	6/1/71-5/31/76
Central Control of Distribution of Organ Blood Flow	U.S.P.H.S. (HL 09154)	45,148	9/1/72-8/31/75
Behavioral Hypertension and Arteriosclerosis: Effects of Nicotine and Carbon Monoxide	Council for Tobacco Res. U.S.A. (CTR 843)	150,000	1/1/72-12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Central Control of Distribution of Organ Blood Flow	U.S.P.H.S. (HL 09154) Renewal Application	250,000 (Approx.)	9/1/75-8/31/80
Behavioral Influences on Cardiovascular Disease (Research Scientist Award)	U.S.P.H.S. (MH 13414)	142,500	1/1/75-12/31/79

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name J. Alan Herd, M.D.

Signature J. Alan Herd Date July 17, 1974

Telephone 617 (Area Code) 734-3300 (Number) 754 (Extension)

Checks payable to

Harvard Medical School, c/o Business Office

Mailing address for checks

25 Shattuck Street

Boston, Mass. 02115

Responsible officer of institution

Typed Name Henry C. Meadow

Title Executive Secretary, Committee on Research and Development

Signature Henry C. Meadow Date 7.19.74

Telephone 617 (Area Code) 734-3300 (Number) 441 (Extension)

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#987 RILEY

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 26, 1974

Grant application No. 987

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Meier and Sommers

Subject: Vernon Riley, D. Sc., Pacific Northwest Research Foundation
New application No. 987
"A small Animal Model for the Study of Factors associated with Hypertension and Cardiovascular Disease".

Seattle Washington

History

An inquiry proposing studies of effects of stress on cardiovascular diseases and cancers in animals was handled as Case No. 153 late in 1972. Formal application was not encouraged.

In May 1974 Dr. Riley visited the CTR offices. Discussions suggested that his system might help evaluate the stress factor in smoking machine exposures. The enclosed application resulted.

Request

Application No. 987 requests $55,979 direct costs for a first year. Indirect costs are "to be negotiated". Support for at least one additional year is suggested.

Documents submitted

1-Letter to Dr. Gardner dated May 24, 1974.
2-Application dated 5/22/74 (arithmetic in budget subject to correction).

Comment

Enclosures are a copy of Dr. Gardner's commentary dated May 21, 1974.

FWN:gh
Enclosures

F. W. N.

FWN

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

PACIFIC NORTHWEST RESEARCH FOUNDATION

Laboratories For Medical Research

DEPARTMENT OF MICROBIOLOGY

May 24, 1974

Dr. William U. Gardner
Scientific Director
The Council for Tobacco Research
110 East 59th Street
New York City 10022

Dear Bill:

The enclosures include reprints that provide background information on the small animal models that have been developed in our Department and elsewhere, together with some of the basic information that has been obtained by these models in approaching both the obvious and the more subtle problems associated with atherscleros is, arterial coronary diseases and some of their known precursory conditions such as environmental stress, essential hypertension, defective renal function, etc.

It is obvious that there are many areas where these small animal models can be brought to bear upon the etiology of cardiovascular disease and associated syndromes. A pertinent example is the comparative role of environmental circumstances, which can be controlled in our laboratories with infinitely greater precision than by other presently known approaches.

As you know from our discussions, we are now in the fortunately unique position of controlling the environment, insofar as mice are concerned, ranging from severe social and physical stress to a tranquil environment that provides a very low corticosterone baseline, which we feel is a good index of biological serenity, and thus prerequisite to freedom from CNS, hormonal, enzymic, and other adverse physiological factors which are either causal, or synergistic with other conditions such as diet, genetics, or exposures to biological or chemical carcinogens or other pathological potentiating substances.

Some of the logical approaches which we visualize as effective experimental means for providing basic information on the etiology and possible modification of atherscleros is, hypertension, renal pathologies, and cardiovascular diseases are outlined in the attachments which are submitted as a preliminary grant application.

The multiple disiplinary approach which we propose is feasible through the combined background experiences of Dr. George Santisteban, a pioneer in developing our understanding of the pituitary-adrenal-thymus-corticoid axis; Dr. Darrel Spackman, a biochemist with a superb background of experience in proteins, enzymes, amino acids, and gluco corticoids. My own background includes laboratory experiences and training in animal and cell biology, virology, experimental chemotherapy, enzymology, and cellular metabolism.

I trust that the enclosures will provide you and your colleagues with an adequately clear indication of the areas where we are both interested and competent to provide basic scientific information that will have application in the prevention and possible reversal of human pathologies that cause such a high mortality.

Sincerely,

1003544274

Vernon Riley, Chairman Dept. of Microbiology

Member, Fred Hutchinson Cancer Research Center

1102 Columbia Street, Seattle, Washington 98104 • (206) 292-2923

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THE COUNCIL FOR TOBACCO RESEARCH--U.S.A., Inc.

110 East 59th Street
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Application for Research Grant Date: 5-22-74

RECEIVED
MAY 27 1974

1. Principal Investigators:

Dr. Vernon Riley, D.Sc., Chairman, Department of Microbiology
Member, Fred Hutchinson Cancer Research Center.

Dr. George A. Santisteban, Ph.D., Senior Biologist, Department of Microbiology.
Professor of Biology, Seattle University.
Affiliate, Fred Hutchinson Cancer Research Center

Dr. Darrel H. Spackman, Ph.D. Senior Research Biochemist, Department of Microbiology.

2. Institution & Adress:

PACIFIC NORTHWEST RESEARCH FOUNDATION
1102 Columbia Street
Seattle, Washington 98104

3. Departments where research will be done or collaboration provided:

Department of Microbiology, PNRF
Department of Biology, Seattle University

4. Short title of study:

A SMALL ANIMAL MODEL FOR THE STUDY OF FACTORS ASSOCIATED WITH HYPERTENSION AND CARDIOVASCULAR DISEASE.

5. Proposed starting date: June 1st, 1974.

6. Estimated time to complete: Two years.

7. Brief description of specific research airms:

A small animal model will be utilized to investigate the etiological role of a variety of experimental parameters in the induction of essential hypertension, renal lesions, atherosclerosis, and other manifestations of cardiovascular disease.

An important aspect of the working hypothesis of this project will be the strict control of environmental factors. This can be accomplished in a practical way only through the use of small animals, preferably genetically defined mice.

Information presently available, through data produced by our laboratory and others, demonstrates the vital role of various forms

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of stress-inducing factors in the promotion of hypertension, which is followed by cardiovascular and other lesions.

The experimental models to be employed in these studies will be designed to effectively control or to introduce stress, ranging from a completely serene situation to a rigorous psycho-social environment that is known to produce hypertension and cardiovascular lesions, and simulates in a controlled way some of the varieties of stress experienced by man in his present society.

Various physical, biological, and chemical factors will be superimposed upon this controlled environment in order to examine their various individual influences, together with possible synergistic effects of stress in combination with chemical and other environmental factors.

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8. WORKING HYPOTHESIS

INTRODUCTION

Recent developments in the epidemiology of essential hypertension and cardiovascular disease place increasing emphasis on the etiologic role of psychosocial stimulation and stress. Some investigators assert that such psychosocial stimulation resulting in a defense alarm response constitutes a significantly more important factor than obesity, diet or exercise (1).

In work carried out in our laboratories and elsewhere (2, 3), there has been induced in an isogenic strain of mice (CBA), sustained arterial hypertension which was accompanied by degenerative changes in the myocardium, coronary blood vessels, aorta and kidneys. This resulted from subjecting the mice to prolonged and repeated social interaction in a special population cage under controlled dietary and environmental conditions. Under these conditions there also occurred an accelerated physical deterioration of the males, a reduction in the size of litters, and a failure of the females to raise their young.

The working hypothesis is that the above observations may be either directly or indirectly attributed, for the most part, to the occurrence of disproportionately elevated, prolonged and repeated defense alarm arousals, with a consequential activation of the sypathetic-adrenal medullary, and pituitary-adrenal cortical systems. Current studies in our laboratories implicate the pituitary-adrenal-thymus axis as a major factor.

The overall objectives of our proposed research are to gain a greater understanding of the biochemical and biological mechanisms whereby psychosocial stress leads to hypertension and cardiovascular disease in mice. An expanded knowledge of such physiological mechanisms in a small animal model will offer obvious advantages to subsequent studies in the human. Additional aspects of our objectives are to develop or improve the methodologies and techniques needed to establish the mouse as an optimal small animal model that may be employed to facilitate studies concerning the etiology and control of cardiovascular diseases and related pathologies.

BACKGROUND

Studies in the past have firmly established that a variety of stressful events experienced by laboratory animals or humans will produce a corresponding elevation of plasma adrenal corticosteroids. The results of controlled injections of corticosteroids has also been investigated. For example the relationship between injections of high, non-physiological doses of corticosteroids and subsequent involution of the thymus is firmly established (4). A concomitant destruction of circulating lymphocytes (T-cells) usually accompanies such thymolytic effects. In considering the question of the significance of these events upon the physiological competence of the host, such experiments have been questioned on the basis that the corticosteroid injections used to produce thymus involution have been selected to produce an approximate 50-fold elevation in plasma corticosteroid and thus far above expected physiological levels (5).

The potential importance of this question bears not only upon the etiology of hypertension and cardiovascular disease but also upon the immunological

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competance of the host and thus upon cancer and many other diseases.

We have now shown, for the first time to our knowledge, that stressful events normally encountered in the routine shipping, housing, and handling of mice, produce remarkable elevations in plasma corticosterone which are accompanied by equally rapid reductions in circulating lymphocytes, and an involution of the thymus (6). Thus physiologically-derived elevations of cortisosterone are capable of producing the results observed heretofor only following injections of rather large doses of corticosteroids. The above results are shown in the following figures.

Figure 1 demonstrates some of the tangible biochemical consequences of inadvertant stress during and following the routine porcedure of shipping mice from the commercial breeder's establishment to the laboratory. Upon receipt of the mice, it may be seen that the circulating corticosterone is elevated about 5-fold above normal (all samples taken at 1:00 P.M.). As shown, this physiological elevation was sufficient to produce a corresponding involution of the thymus. A significant decrease in circulating lymphocytes (T-cells) was also observed. After the mice are placed in a reassuring, uncrowded environment, the measurable effects of the shipping stress gradually subside. Corticosterone and lymphocyte levels are back to normal within a few days although a normal thymus weight is not regained until 1 1/2 to 2 weeks.

Figure 2 demonstrates further that the routine handling of experimental animals can have a striking influence on physiological parameters that may relate to hypertension. As shown here, the excitement induced in mice during the moving, capturing, and handling which accompanies routine orbital bleeding and other laboratory procedures, produces an alarm reaction with an increase in plasma corticosterone. A corresponding rapid loss in circulating lymphocytes (down to 50% after 40 minutes) and a loss in thymus weight were also observed. The cluster of disconnected points at the bottom of the curve in Figure 2 show that if ten mice in a given cage are bled rapidly, within 3 1/2 minutes, the blood samples show no evidence of corticosterone elevation. However, if the blood samples are obtained from similarly disturbed mice at longer, spaced intervals, a rising curve exhibiting the steadily increasing corticosterone concentration in the blood, beginning at 4 minutes, is obtained.

It is important to note that these corticosteroid increases are physiologically produced and thus can be considered a "normal" range that the host is repeatedly exposed to during experimental manipulation, and handling during observation.

AIMS AND OBJECTIVES

In the studies proposed in this application, we expect to gain a greater understanding of the biochemical and biological mechanisms whereby psychosocial stress leads to hypertension, atherosclerosis, and other cardiovascular diseases. Following are some of the studies which we propose to carry out.

1. Establish non-stress baseline values for adrenal corticosteroids as a function of mouse strain and sex.

Some of this information has already been established in our laboratories. We have determined the diurnal variations in BAF_1 female mice. Still needed are similar determinations in male BAF_1 mice and in other strains of mice. A micro fluorimetric assay procedure for plasma

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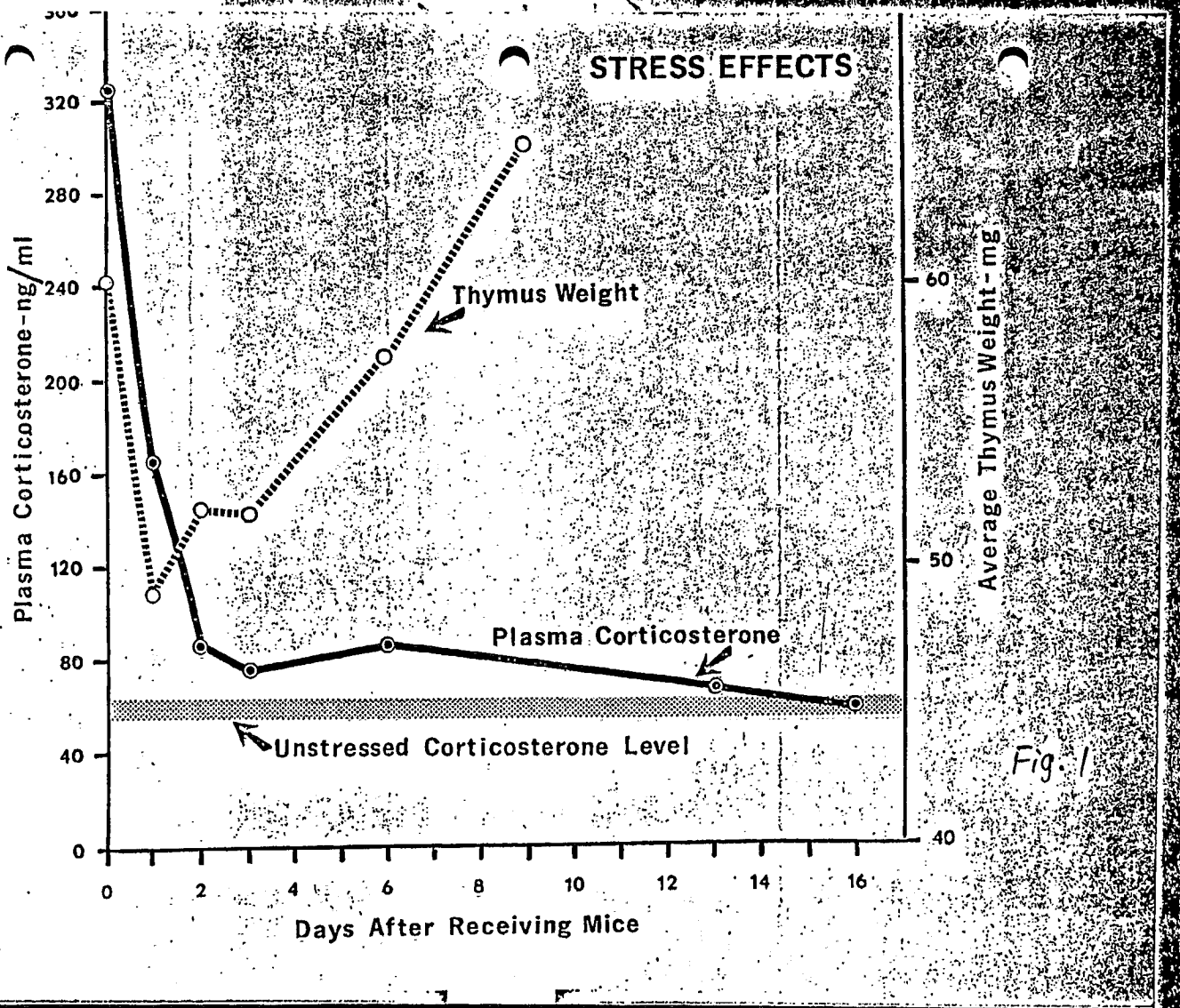

Fig. 1

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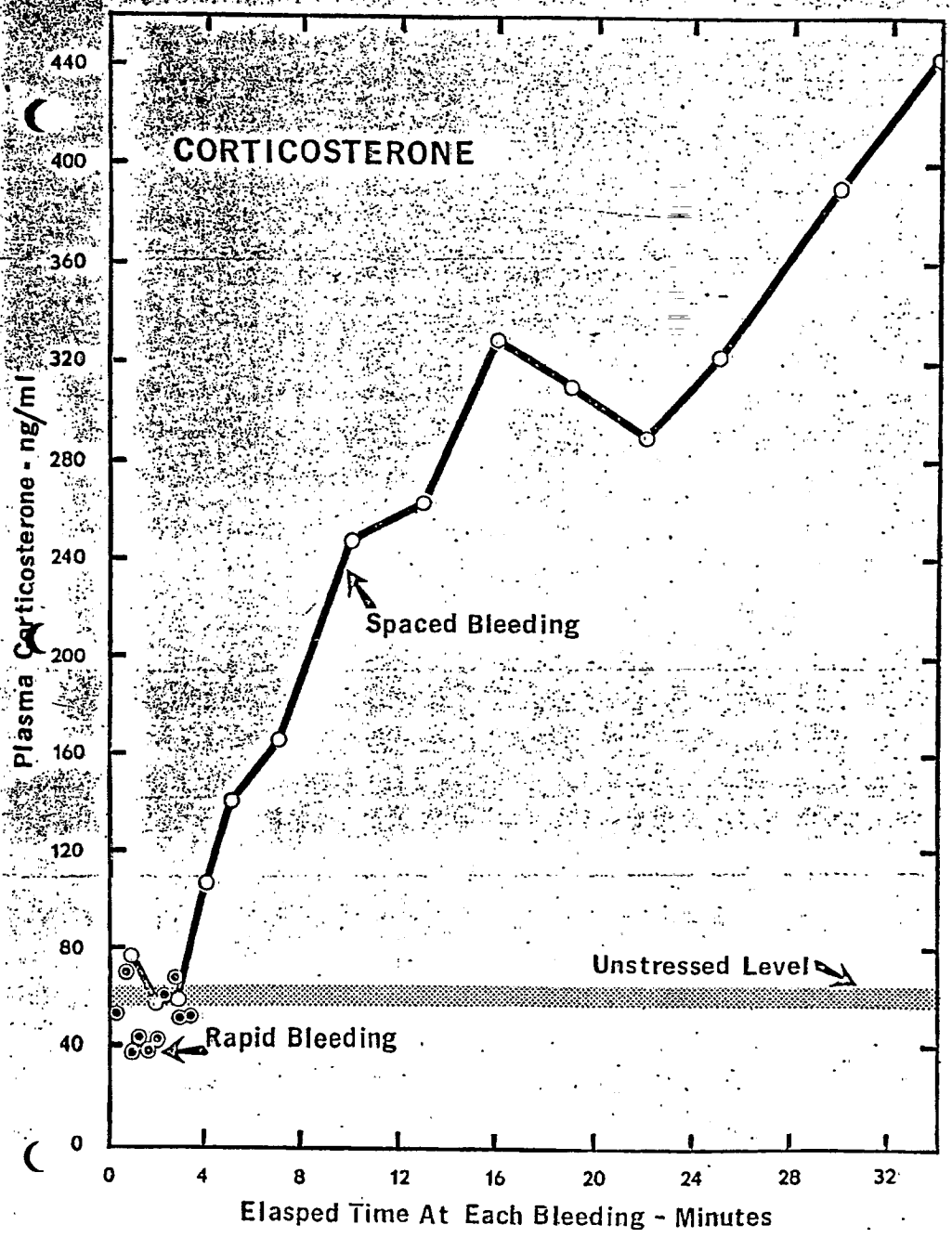

Fig. 7

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corticosterone was developed in our laboratories specifically for use with the small samples of blood which can be obtained serially from mice by using the rapid and convenient orbital bleeding procedure.

2. Expand the psychosocial stress studies initiated in our laboratories by Dr. Sanisteban (2, 3), using specially designed population study cages, and correlating such "normal" stress factors with progressive essential hypertension and arteriosclerotic changes in the heart, kidney and aorta.

Our search for an explanation of the mechanisms responsible for the above phenomena has led us into consideration and examination of the hormonal and neurobiological aspects of the defense alarm responses. Evidence of increased activation of the sympathetic nervous system and both components of the adrenal gland has been obtained. Thus in our chronically socialized mice there have been shown to occur increases in blood levels of catecholamine biosynthetic enzymes, epinephrine and norepinephrine (2, 7). Also, we have observed involution of the thymus and depletion of adrenal cholesterol and ascorbic acid (8). Preliminary results also show a definate elevation in plasma levels of corticosteroids during social stress.

These results are in accord with human studies which have shown that persons undergoing typical defense alarm stimulation have increased levels of 17-hydroxycorticosteroids (17-OHCS) (9); whereas a fall in 17-OHCS levels occurs in response to soothing, relaxing inputs (10). Clinical conditions characterized by high blood pressure and high levels of 17-OHCS include Cushing's Syndrome, in which there is an excessive production of glucocorticoids (11), and Conn's Syndrome, where there is a high blood level of aldosterone (12). Treatment of adrenal cortical deficient patients with large amounts of 17-OHCS leads to an increase in blood pressure. Conversely a drop in blood pressure is characteristically observed in Addison's disease and following adrenalectomy (13). Finally, hypertrophy of the adrenal cortex is often observed in animals undergoing chronic stimulation (14). Thus it appears that the pituitary-adrenal cortical system is intricately involved in the induction of hypertension by acute and chronic social stress.

Our studies on the sequence of pathological changes occurring in the heart, blood vessels, and kidney in socially stimulated hypertensive mice (2), support the reports that at high blood pressures the walls of the small arteries deteriorate and tissue nutrition fails due to a developing obstruction in blood supply (15, 16). In the heart, the vascular damage impairs the blood supply to the myocardium, which, with the progressive elevation in blood pressure, has a progressively increasing work load. In the brain there results an ever-increasing risk of death or disablement from brain hemorrhage. In the kidney the vascular derangement leads to the development of glomerulonephritis and, less frequently, to pyelonephritis (17, 18). With increasing obstruction, there is a progressive elevation of blood pressure. If the rise is rapid and severe, it will be associated with an increased renin production and the resulting elevation in blood pressure can lead to levels in excess of 200 mm Hg in a matter of weeks. This can be fatal within a few months unless it is controlled by surgery or by the use of drugs (17, 18, 19).

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3. Determine the changes in plasma and tissue amino acids produced by elevations of adrenal corticosterone. These amino acid studies would be carried out following experimental injections of corticosteroids as well as a part of long-term psychosocial, hypertension studies using the population study cages.

Adrenal corticosteroids have several specific influences on carbohydrate and amino acid metabolism, including a gluconeogenesis from amino acids. The latter may result from an increased availability of amino acids because of the inhibiting effect of adrenal cortical steroids on amino acid incorporation into proteins. Specifically, protein synthesis is inhibited in muscle and other tissues at high hormonal levels. Presumably, adequately high levels would be obtained under stress. Since protein degradation in the same tissues is continuously occurring, there is a net loss of amino acids resulting in tissue wasting. Metabolic oxidation of these amino acids would thus lead to an overall negative nitrogen balance. Under such conditions plasma free amino acids would be expected to be elevated.

The foregoing mechanisms may explain the observations made by Henry, et.al. (2) that in psychosocial stress there seems to be a more rapid loss or wasting of body proteins (thin skin, etc.). Such mechanisms may also be associated with the elevated blood pressure and obesity observed with Cushings Syndrome (glucocortcoid elevation) (11), and Conn's Syndrome (aldosterone elevation) (12).

4. Determine whether any cortisol, in addition to the predominant corticosterone, is present in mouse plasma, either normally or as a result of severe chronic stress.

It has been established that the only circulating 17-hydroxycorticosteroid in the rat is corticosterone (20). Cortisol apparently is not present. We intend to make such a determination in the mouse. This information is of some importance, for on the one hand, cortisol gives only one-fourth the fluorescence in assay procedures as does corticosterone, but on the other hand, cortisol is many times more active than corticosterone in most of its physiological effects, including thymus involution. Thus small amounts of cortisol would be difficult to detect during assay and yet, if present, could account for a significant part of the total physiological effects observed in association with stress.

5. Initiate studies to answer the question as to whether the control of diet can retard or prevent the progressive hypertensive and cardiovascular changes resulting from psychosicial stimulation and stress.

Our plasma and tissue amino acid studies may give valuable clues in this direction. For example, if certain essential amino acids were to be found in low concentration, dietary supplementation could perhaps improve overall protein formation and thus improve the overall general health of the host. Ratios of essential to non-essential amino acids also can be a useful index of general nutrition (21).

6. Following the establishment of optimal conditions for measuring the various parameters associated with progressive development of hypertension and cardiovascular and renal lesions, this small animal model and its physical devices may be employed in a variety of experiments to examine the following questions.

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a) How do pertinent dietary alterations influence the onset, progression, control, and possible reversal of atherosclerosis?

b) Having established the influence of controlled social stress upon hypertension and the pathological sequalae, what are the synergistic influences of adding the effects of tobacco smoke, tar, CO, NO_2, nicotine, caffeine, etc., upon the usual course of cardiovascular disease? It may be noted that the population cage complex can be designed for gas and aerosol intrusion without introducing the concomitant factor of stress associated with abnormal confinment or restraint.

c) Is there a synergistic role of benign or pathogenic viruses and other infectious agents in promoting atherosclerotic and cardiovascular and renal pathologies?

Current studies in our laboratories have demonstrated the capabilities of a benign virus for inducing plasma corticosterone elevation, lymphocytopenia and thymus involution. These influences appear to be indirectly caused by physiological stress by way of the pituitary-adrenal-corticosteroid pathways, rather than by a direct viral attack upon thymus tissue. Inasmuch as such physiological effects are also incriminated as etiological factors associated with the experimental psychosocial stress-mediated hypertension, renal lesions, and cardiovascular pathologies observed in mice, and presumed to function in human beings, it would be appropriate to examine the possible role of such infectious agents in this category of disease. Figure 3 illustrates our observations of the induction of high levels of plasma corticosterone following the intentional infection of mice with a benign, ubiquitously distributed murine agent, the LDH-elevating virus.

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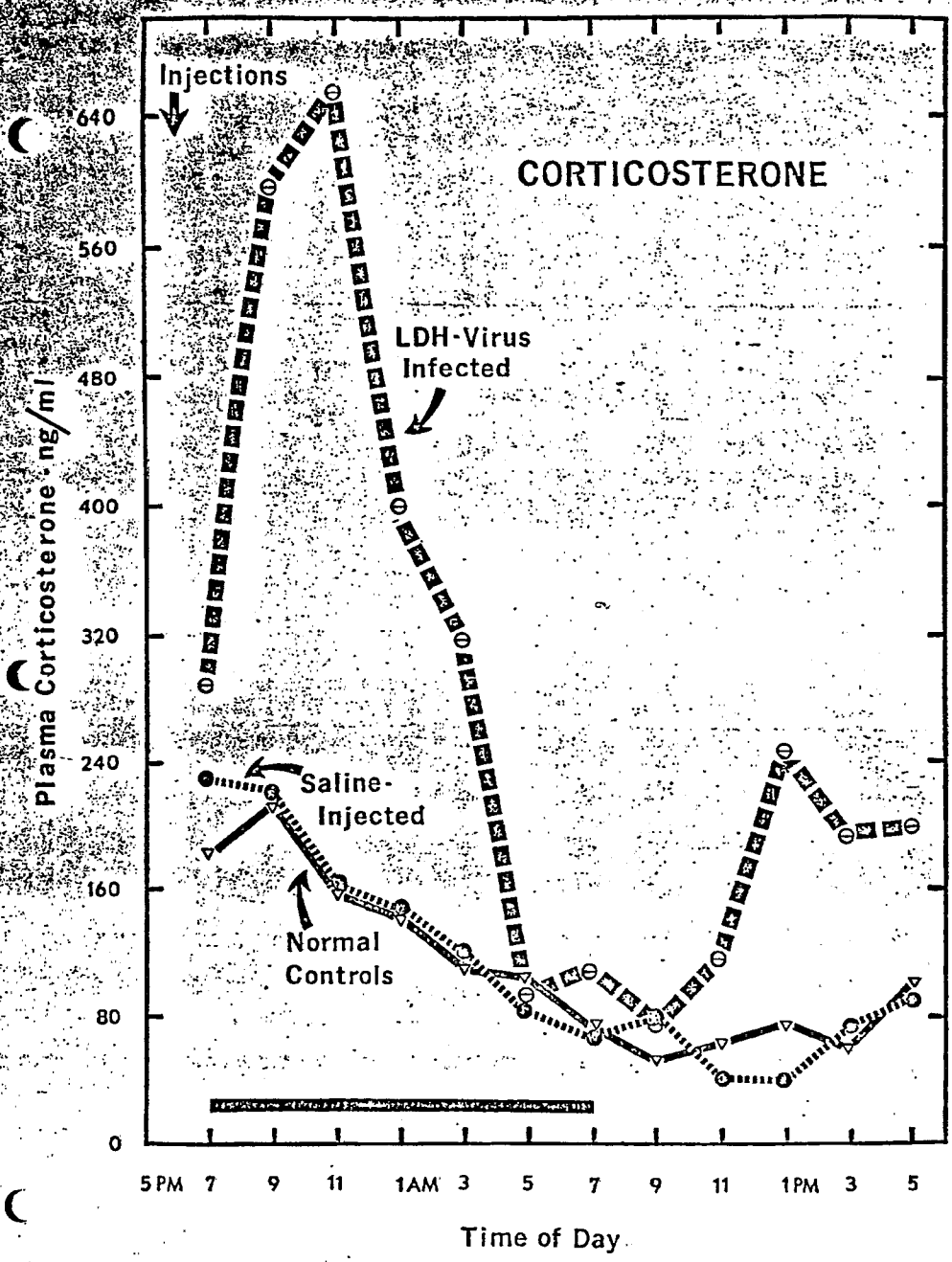

Fig. 3

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9. EXPERIMENTAL DESIGN AND PROCEDURES

General descriptions of contemplated experimental designs and procedures are included in the foregoing section, and in the attached reprints and abstracts. Following, are more detailed descriptions of some of the specific procedures that we plan to employ in carrying out the basic intent of these proposed studies. The several types of controlled experimental environments, which we have at our disposal for the study of such important daily effects upon this small animal model, are also described here in some detail. It is our experience that such controlled experimental circumstances are of vital importance in the critical evaluation of the ultimate significance of various other factors suspected of contributing to cardiovascular disease processes.

[A provocative example of comparative environmental stress, and the resulting incidence of disease, has been noted in our laboratory during studies on the incidence of mouse mammary carcinoma. Under moderate chronic but intermittant environmental stress, C3H female mice carrying the Bittner virus develop the usual 75 to 80 % mammary carcinoma by 15 months; while identical mice maintained under special environmental conditions of minimum stress have shown 0 % tumors during the same period, in current experiments in our laboratories (Riley, V., unpublished data).]

HOUSING, CARE AND STORAGE OF MICE

Protective Ventilated Shelves for Experimental Animal Storage with Minimal Stress. A simple facility has been devised for the protection of experimental animals from both external and intra-laboratory contamination. Additional important features of the facility include protection of laboratory personnel from potential hazards associated with infectious aerosols and allergenic proteins present in most animal storage and work areas. The principle of the design of this facility is that of library wall-type, or free-standing shelves back to back, enclosed by sliding or hinged plexiglass doors. Standard plastic or metal cages of appropriate size are employed in the usual way. Various types of laminar flow ventilation are feasible; however, an effective and simple arrangement presently employed is to attach an exhaust duct to one end of the shelf unit through a suitable plenum chamber, with removable filters installed at the opposite ends for the entry of clean air, usually drawn directly from the room. The contaminated air is exhausted via a window or roof fan, or other appropriate means so that air in contact with the animals never recirculates in the animal facility. Nine years experience with this system has established that it is possible to work simultaneously with a variety of viruses and other infectious entities in the same room without cross-contamination. Greatly improved health of the experimental animals has been observed, with elimination of common rodent respiratory diseases and infantile diarrhea. Experience has also shown that this housing arrangement also imposes a minimum stress environment upon the mice that are housed therein. The improved space efficiencies and odor-free characteristics of this facility permit ordinary animal rooms to be converted to biological laboratories with a more effective and convenient utilization of research space. Features and some construction details are shown in Figure 4.

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Figure 4

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Population Study Cage for the Study of Psychosocial Stress. This consists of a system of interconnected Lucite boxes which allows free intercommunication but also forces the animals to come into contact with each other in a central area which thus becomes a "behavioral sink", as described by Calhoun (22). In the central area there is a hexagonal pen topped with a quarter-inch wire mesh cover from which are suspended a feeding basket and tubes from two watering bottles. Six plastic runways (1 1/2" X 6") radiate from each side of the pen, each leading into a standard "shoe box" type cage. Each box is covered by a removable quarter-inch wire mesh lid. There are one and one half inch plastic tubes interconnecting all adjacent boxes. In this system the animals may move from box to box by way of the peripheral runways and into the central pen from any of the peripheral pens. Studies of the behavioral and adrenocortical and blood pressure responses of animals kept in this life system show that the animals in these pens develop a sustained hypertension (2), and an elevated adrenocortical stimulation (8). These responses are particularly marked in males. It has already been shown that animals raised in isolation are more sensitive and more likely to develop arterial hypertension when placed in this apparently socially stressful situation. The construction of a population study cage is shown in Figure 5.

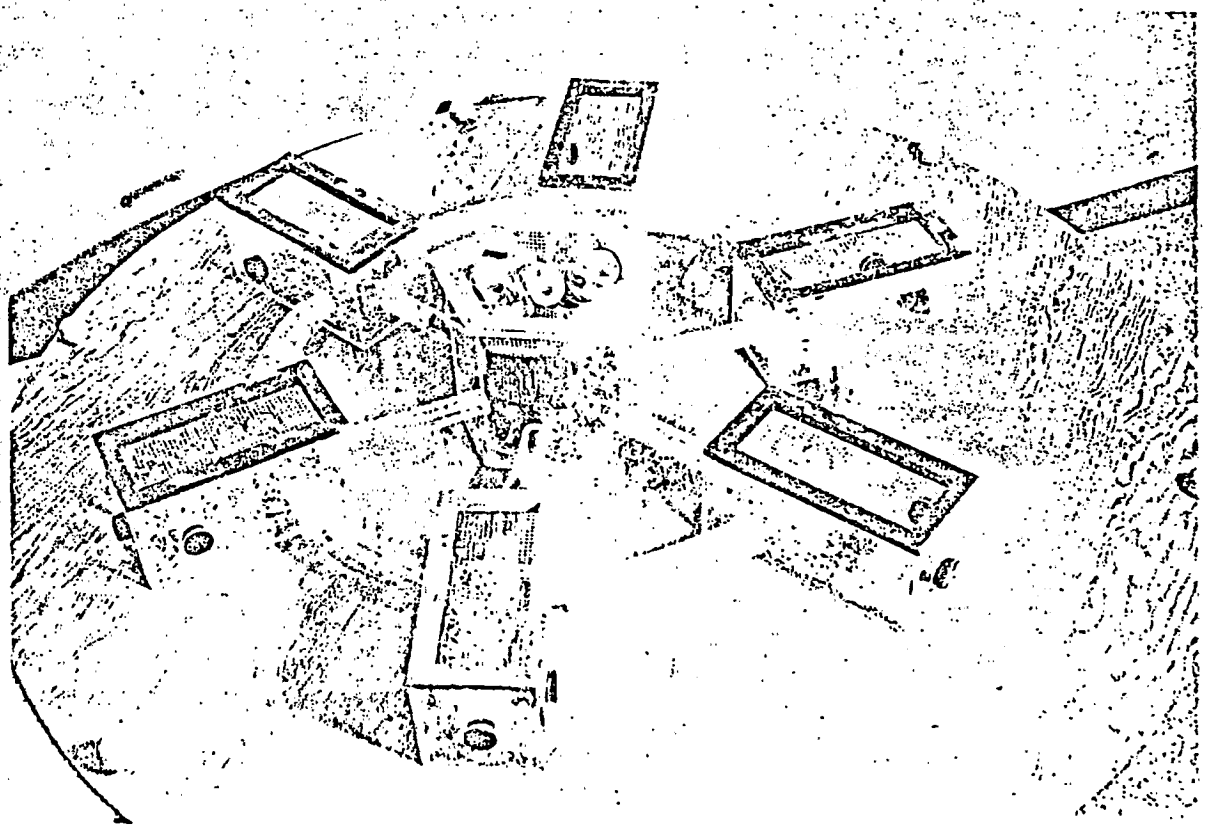

FIGURE 5

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ASSAY PROCEDURE FOR PLASMA CORITOSTERONE IN MICE

Introduction. The micro fluorimetric assay procedure currently in use was developed specifically in our laboratories for use with the small samples of blood which are available from mice, and is based on the procedure described by Glick, et at (Endocrinology 74:653-655, 1964). The orbital bleeding procedure which is used exclusively in routine bleedings in our laboratory, provides 50 to 100 µl quantities of plasma. Our procedure has been designed to use 50 µl plasma samples and thus either single or duplicate assays on the blood from a single mouse may be performed as desired. The method gives accurate and dependable results using sample quantities of corticosterone that may range from 0.5 to 50 ng (10 to 1000 ng/ml). Since there is some normal biological variation in individual mice, many of our experiments utilize groups of 5 to 10 mice. The corticosterone assays are run in duplicate, triplicate, or quadruplicate on plasma pools obtained from such groups.

Washing of Glassware. This is a critical step in any fluorimetric procedure and particularly so in a micro assay. The procedures which we use have been chosen after extensive evaluation to provide thorough cleaning (and thus minimum interference) and yet permit reasonably rapid routine procedures using 12 or more assays per batch. All glassware is first rinsed or soaked in Liquinox cleaning solution. Glassware that has not been in contact with plasma or corticosteroid standards is then rinsed in tap water 10 times, deionized distilled water 3 times, and acetone (from a polyethylene wash bottle) 3 times. After air drying (not to exceed 20 minutes), the items of glassware are covered with Saran film or enclosed in containers, to prevent contamination from airborn particles and vapors. Since we have determined that "disposable" Pasteur pipetts and culture tube cuvettes must be prewashed before use, we routinely clean and use these items repeatedly. Non-heparinized bleeding tubes are washed on a neoprene adapter in lots of 24 and then heparinized with sodium heparin. The extraction tubes (Corning 2-ml volumetric flasks with glass stoppers) and other glassware which comes in contact with plasma or corticosteroid standard solutions are rinsed in Liquinox cleaning solution followed by water, drained, and are then immersed in a stainless steel basket in chromic acid cleaning solution (made by adding 70 ml of a saturated solution of sodium dichromate to two liters of technical grade sulfuric acid) a minimum of two hours but usually overnight. They are then rinsed and stored as described above.

Reagents. All organic reagents are purchased as "Reagent Grade" and are distilled before use. This includes acetone, absolute ethanol, chloroform, methylene chloride and carbon tetrachloride. Inorganic reagents are of Reagent Grade, and where water is used, it is of deionized-distilled quality.

The acid-alcohol reagent which is used to develop fluorescence, forms a critical part of the overall procedure. A 2:1 volume/volume ratio of sulfuric acid to ethanol has been found to give maximum fluorescence with corticosterone and minimum fluorescence with interfering substances found in plasma. It is mixed in a glass-stoppered Erlenmeyer flask which is partially immersed in a crushed ice bath. It is important to keep the reagent cold during mixing and subsequent storage to provide low background blanks.

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This reagent is mixed fresh daily.

Procedure. Aliquots of 50 µl of plasma or working standards of corticosterone (or cortisol) are pipetted into reaction flasks followed by 50 µl of water. Volumes of 650 µl of chloroform are then added to each flask from a repetitive pipette dispenser. The flask is stoppered and mixed on a vortex mixer (in groups of four) for 60 seconds to extract the corticosterone. After centrifugation (1 min) and removal of the aqueous layer by controlled vacuum aspiration with a glass capillary, 100 µl of 0.1 N NaOH is added, the flasks vortexed for 15 seconds, centrifuged, and the aqueous layer again removed. An aliquot of 500 µl of the chloroform solution is then transferred to another reaction flask containing 400 µl of the acid-alcohol reagent (prepipetted before the extraction procedure is started). After 30 seconds of vortex mixing, followed by centrifugation, the upper chloroform layer is removed by aspiration and the total acid-alcohol volume transferred to a 6 x 50 mm Pyrex culture tube cuvette. The fluorescence is allowed to develop at room temperature (22 to 23°). Readings are taken at 80 min and again at 90 min, starting from the time when the chloroform solution is mixed with the acid-alcohol reagent (in a Turner Model 111 fluorometer fitted with a micro cuvette adaptor). A general purpose lamp (#110-850) plus Turner 2A and 47B filters provide a 436 nm excitation beam. For the emission wavelength a 2A-12 filter is used.

Calculations. We have determined that a fluorescence reading (corrected for blank) that is linear with concentration is obtained when samples of corticosterone varying from 50 to 800 ng/ml are assayed by this procedure. Therefore fluorescence units from plasma samples are converted to concentration units by multiplying by the constant derived from the foregoing relationship. In addition, the standardization is constantly monitored by analyzing duplicate samples of corticosterone standard with each 12-sample batch that is processed. Two of the samples are water blanks which are then used to correct the other sample values.

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PROCEDURES USED WITH AMINO ACID ANALYSIS

Mice. Hybrid BAF (C56Bl/6 x A) F_1 mice will be used initially for these studies. Other strains, such as BDF_1 and C3H, are carried in our laboratories, and will be used as required. Female mice between 6 and 12 weeks of age, weighing 18 to 22 grams will be generally employed. All mice received from breeders are placed under close observation for a period of 2 weeks for signs of any respiratory or other diseases or other evidence of ill health before being used in experiments.

Blood Sampling Techniques. Inasmuch as the circulating amino acid pool *in vivo* represents a host equilibrium between influx, clearance, enzymic alterations, and perhaps other physiological events, it is apparent that losses of certain amino acids will occur in blood removed from the host if the samples are not obtained, processed, and stored under conditions designed specifically to prevent such losses. For example, rapid changes would be expected to occur in plasma asparagine concentration during the administration of asparaginase, which has a specific effect on this amino acid. Appropriate sampling procedures have, therefore, been designed to minimize amino acid alterations during bleeding and the further processing of plasma samples for amino acid analysis (50).

Essential principles of these procedures include keeping the blood samples cold during processing, reducing sample preparation time to a minimum, and precipitation of plasma proteins, including all enzymes, as rapidly as possible after the withdrawal of the blood sample.

Blood samples of about 0.2 ml per mouse are removed form 4-hour fasted mice by the orbital bleeding technique (51) using pre-chilled, disposable, heparinized bleeding tubes. The actual bleeding procedure requires only a few seconds; the tubes are then quickly stoppered and plunged into an ice bath, chilling the blood immediately to 0°C. The blood samples are centrifuged at 0°C, and the cold plasma samples are transferred immediately into pre-weighed, disposable tubes containing the protein precipitating agent. Thus all plasma proteins, including exogenous asparaginase, are removed from solutions at the earliest possible moment and all enzyme activity is terminated.

Urine and Tissue Samples. Urine samples are collected by induced voidance as needed and also in special metabolic cages for 24-hour collections. Organs and tissue samples will be removed as rapidly as possible from decapitated mice. These samples will either be homogenized, deproteinized, and further prepared for analysis immediately, or will be quick-frozen on dry ice for short periods of time until such preparation can be conveniently carried out. Healthy young adult mice will be used as a source of normal tissues, organs, and fluids. For tumor tissue, firm, non-necrotic portions of the rapidly growing tumor will, in general, be selected.

10035442 91

Methodological Improvements

Extensive developmental work has been carried out by the Principal Investigator in the general area of amino acid analysis. The original description of the "automatic" amino acid analyzer was published in 1958 by Spackman, Moore, and Stein (24, 25); associated methods of chromatography

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for amino acids and related compounds, has been the picric acid method (38). This method is objectionable, however, because of the length of time and significant manipulation required for the operation. Other widely used methods have made use of sulfosalicylic acid (SSA) (39-42) although trichloroacetic acid, phosphotungstic acid, and periodic acid have also been used for this purpose. Another method makes use of low sample pH and ultracentrifugation for a partial removal of sample proteins (40). Ultrafiltration has also been used (38, 43).

Because of the limited volume of mouse plasma available for amino acid analysis, we have developed methods which allow all of the plasma sample to be utilized in producing the final deproteinized sample. For these purposes sulfosalicylic acid has been selected as the protein-precipitating agent of choice. Sulfosalicylic acid solutions have been used at several different concentrations and total volumes for the deproteinization of physiological fluid samples. Our investigations show that significant errors can be inadvertently introduced in most of these methods due to failure to obtain total recovery of the amino acids at each step in preparing the sample. We have, therefore, developed a reproducible, effective method designed to eliminate these errors. It utilizes plasma samples of 1 ml or less and does not require volume reduction of the deproteinized sample prior to analysis on the amino acid analyzer. The pH of the sample is carefully controlled in order to give the most efficient precipitation of proteins during preparation without destruction of labile amino acids, and to give optimum, reproducible chromatographic placement of pH-sensitive amino acids during the analysis. After the initial precipitation and removal of supernatant, the remaining precipitate is suspended and extracted three additional times to give better than a 99% recovery of all free amino acids originally in the plasma sample.

Inasmuch as the circulating amino acid pool in vivo represents a host equilibrium between influx, clearance, enzymic alterations, and perhaps other physiological events, it is apparent that important changes in certain amino acids will occur in blood removed from the host if the samples are not obtained, processed, and stored under conditions designed specifically to prevent such alterations. For example, rapid changes would be expected to occur in asparagine concentration in the presence of asparaginase. Appropriate sampling procedures have, therefore, been developed to minimize amino acid alterations during both the collection of blood and the processing of plasma samples for amino acid analysis.

Essential elements of these procedures include keeping the blood samples cold during processing, reducing sample preparation time to a minimum, and precipitation of plasma proteins, including all enzymes, as rapidly as possible after the withdrawal of the blood sample.

One or more separate blood samples of approximately 0.2 ml volume are removed from each mouse by the orbital bleeding technique using disposable, heparinized bleeding tubes. The actual bleeding procedure requires only a few seconds. After filling, each tube is quickly stoppered and plunged into an ice bath, chilling the blood immediately to 0°C. The blood samples are centrifuged at 0°C, and the cold plasmas are transferred immediately into pre-weighed, disposable tubes containing the protein precipitating agent. Thus, all plasma proteins are removed from solution at the earliest possible moment and the enzyme activities are terminated.

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were also described (26). Further developmental studies for increasing the sophistication of the instrument were carried out at the Spinco Division of Beckman Instruments, at the University of Washington, and at Pacific Northwest Research Foundation. Following the introduction of the automatic amino acid analyzer, improvements have been made in several ways through the efforts of several investigators. Analyses have been accelerated, thus shortening the time required for each individual assay (27-31); micro methods have been developed requiring small quantities of sample (32); and spherical-bead ion exchange resins have permitted higher flow rates and improved resolution (33, 34).

Recent improvements have been made in elution procedures utilizing lithium buffers rather than sodium buffers for the analysis of acidic and neutral amino acids (27, 35). By the use of these buffers, asparagine, for the first time, has been separated from glutamine in an overall analysis procedure.

Published procedures were found to permit only a marginal resolution of asparagine, glutamic acid, and glutamine from each other; this was also true of the resolution of serine-threonine. These procedures were also inadequate for an acceptable separation of the amino acids cystine, methionine, homocitrulline, cystathionine, isoleucine, and leucine. Homocitrulline, which is present in both urine and plasma, is completely obscured by the other amino acids in this region.

Because of these inadequacies we have developed a new, improved method of analysis using lithium citrate buffers (36, 37). A three-buffer sequence has been adopted for optimal resolution of all of the acidic and neutral amino acids normally found in physiological fluids. The resulting improved resolution of asparagine-glutamic acid-glutamine has been especially useful in our amino acid deprivation studies of leukemia using asparaginase and glutaminase.

An improved analytical method has also been devised for the chromatography of the basic amino acids found in physiological fluid samples, particularly blood plasma (36, 37). With this revised method, excellent resolution of all known basic components is achieved. A significant advantage of this new system for the basic amino acids is that it has allowed us to resolve and demonstrate the presence of a new amino acid, E-N-methyllysine, in mouse plasma (5, 11).

Appropriate changes in valves, timers, and other control equipment have been made to incorporate the above modifications into the overall, routine automatic analysis of physiological fluid samples.

An increase in sensitivity has been achieved with the Model 120B by using cuvettes which have a flow path of 20 mm in the photometer. This has been facilitated through the use of new non-darkening incandescent lamps with a tungsten-iodide filament (37).

The procedure most widely used in the past for the deproteinization of physiological fluids and tissue extracts, that are to be subsequently analyzed

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In analyzing tissues for free and non-protein bound amino acids, one significant problem is what to do with glutathione. Although absent in plasma and urine, both the reduced (GSH) and the oxidized (GSSG) forms are present in various tissues and red blood cells. They chromatograph as separate peaks, and interfere with the chromatography of other amino acids. The GSSG peak is broad and overlaps several amino acids using either the sodium citrate buffer system (25, 44) or the lithium citrate system (D.H. Spackman, per. obser.) for chromatography of acidic and neutral amino acids. In the lithium buffer system, used for our analyses, GSH partially overlaps aspartic acid. Although GSH is normally absent from mouse plasma, any hemolysis of red cells (which contain GSH) before separation of the plasma will introduce this peptide into the plasma fraction. This is potentially serious in asparaginase therapy studies where accurate measurements of aspartic acid are needed. An effective method has been described (25, 44) for removing GSH and GSSG by conversion to the sulfonate, but this procedure is rather time-consuming and thus does not readily lend itself to schedules involving the preparation of multiple samples. Preliminary experiments have been undertaken and it is expected that we will be able to accelerate and improve this aspect of sample preparation.

Very few studies have been made to systematically investigate the detrimental effects of delays or of various procedures upon the integrity of the sample to be analyzed. Neither have critical studies been completed to determine the effects of both short-term and long-term storage of samples awaiting analysis. One report has shown that both asparagine and glutamine decrease in concentration following storage of frozen, non-deproteinized plasma samples, and that cystine is completely lost after seven months storage of such samples (38). Our own results show that most of the cystine is lost from plasma samples in as little as 2 or 3 days of storage at 4°. If asparaginase is present in the plasma, one hour of storage at 4°, or 24 hours at -20° will cause severe losses (50 to 95%) of both asparagine and glutamine.

There is little doubt that the amino acids in deproteinized samples are much more stable than they are in plasma. A few analyses have been made in our laboratories to determine time-course changes in the acidic and neutral amino acids in samples prepared from mouse plasma. These samples were analyzed when freshly prepared and again after 21 months of frozen storage (at -20° for 12 months and -80° for 9 months). The average changes from four such samples are shown in Table IV. These data show that while most amino acids are quite stable, some changes do take place. The greatest percentage change of those listed is for asparagine.

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10. FACILITIES AVAILABLE

Adequate physical facilities, composed of 16 modules of laboratory space, are available to the Department of Microbiology where these studies will be carried out. A suite of five rooms (approximately 1,000 sq. ft.) are available for experimental animal studies. This includes two animal rooms ("clean" and virus-infected) with special ventilated shelves for securely housing normal and infected mice and other experimental animals (see separate description of these facilities). Adjacent to the animal quarters are two rooms for laboratory operations. These rooms connect with either the "clean" or virus-infected animal rooms, providing an airlock and contamination barrier. An additional room is available for the safe storage and processing of food, bedding, cages, etc.

Six additional laboratories are available (approximately 2,000 sq. ft.) for carrying out the specialized research activities described in this application. A walk-in cold room, photography darkroom, and other standard laboratory facilities are available, including facilities for glass washing and sterilizing. Services of a fully equipped instrument shop are available at the adjacent Medical Center.

Available office space includes a two-module room for Departmental activities, and additional rooms for administrative, accounting, and secretarial operations. The Department provides a small reference library, including current journals that are related to cancer research.

Comprehensive laboratory facilities of a modern 500-bed hospital can also be called upon for special needs.

Two fully automated amino acid analyzers (Beckman Models 120B and 120C) equipped with digital integrating computers have been purchased for the use of the Department. These are essential instruments for studies such as proposed in this project.

Two Warburg manometric instruments, complete with calibrated glassware, are available for enzymic or other metabolic studies on normal and neoplastic cells.

A laboratory equipped for cell culture operations is a part of the Departmental facilities. Electron microscope facilities are available in the Foundation.

Following are additional items of equipment available for these studies:

Cary Recording Spectrophotometer Model 11
Cary Recording Spectrophotometer Model 15
International Refrigerated Centrifuge
Spinco Ultracentrifuge
Corning pH Meter (4 digit)
Orion pH Meter (5 digit)
Photographic Equipment
Coulter Counter, with Plotter
Incubators
Tissue Culture Inverted Microscope
Research Microscopes (several)
Zeiss Photomicroscope
Automatic Pipettes
Mettler Balances (various types)
Microtiter Equipment

11. Additional facilities required: NONE.

10035442 98

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12. BIOGRAPHICAL INFORMATION

(Give the following information for all professional personnel listed on page 2, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
RILEY, Vernon T.	Chairman, Department of Microbiology	REDACTED
PLACE OF BIRTH (City, State, Country)	**PRESENT NATIONALITY (Non-U.S. citizen indicate kind of visa and expiration date)**	**SEX**
REDACTED	REDACTED	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Spokane College, Spokane, Washington	A.B.	1935	Biology
George Washington University			Biology
University of Paris (Sorbonne), France	D.Sc.	1966	Microbiology

HONORS

Doctoral Thesis, Tres Honorable, (Sorbonne); Fellow, New York Academy of Sciences; Chairman, International Pigment Cell Conferences; National Member, Society for Experimental Biology and Medicine.

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Basic physiological precesses involving cell biology, enzymology, and virology.	Collaborator--Department Chairman

RESEARCH SUPPORT *(See instructions)*

National Cancer Institute General Support Grant, Pacific Northwest Research Foundation, Department of Microbiology.

NCI CORE GRANT for the Fred Hutchinson Cancer Research Center.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project, including teaching and clinical responsibilities. List all or most representative publications.*

Chairman, Department of Microbiology, Pacific Northwest Research Foundation, Seattle, Washington, September 1967 to present.

Member, Fred Hutchinson Cancer Research Center, Seattle, Washington June 1973 to present

Full Professor, Experimental Animal Medicine, University of Washington School of Medicin Seattle, Washington (Affiliate), June 1971.

Head of Section, and Head of Laboratory, Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Rye, New York, 1954-1967; Assistant, 1954-1960, Associate, 1960-1967.

Biologist, National Cancer Institute, National Institutes of Health, U.S.P.H.S., Bethesda, Maryland, 1943-1953.

Senior Research Assistant in Chemistry (Oceanographic Laboratory), University of Washington (Fermentation Research), 1937-1940.

Assistant Bacteriologist, Hollister-Steir Laboratories, Spokane, Washington 1935-1936.

The above experiences included 5 years training in quantitative virology with W. Ray Bryan (NCI), and 5 years training in enzymology and cellular metabolism with Drs. Dean Burk, Mark Woods, Jesse Greenstein, and Otto Warburg.

The principal investigator was a visiting scientist with Prof. Charles Oberling, Villejuif, France, 1959; and with Prof. Raymond Latarjet and Dr. Robert Cramer, Laboratorie Pasteur de l'Institute de Radium, Paris, 1960.

The principal investigator received his doctorate in microbiology under the Nobel laureate, Andre Lwoff, Pasteur Institute and University of Paris.

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Riley, V., Spackman, D.H., and Fitzmaurice, M.A.: Influence of asparaginase and glutaminase upon free amino acids in normal and tumor-bearing mice. Abstract, International Symposium on Asparaginase, Paris, November 28-30 (1970).

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Riley, V.: Protective ventilated shelves for experimental animal storage. Proceedings, 23rd Annual Session, American Association for Laboratory Animal Science, St. Louis, October (1972).

Riley, V.: Distribution and characteristics of the LDH-elevating virus. Proceedings, 23rd Annual Session, American Association for Laboratory Animal Science, St. Louis, October (1972).

Riley, V., and Spackman, D.: Amino acid profiles in melanoma tissues and host plasmas. Yale Journal of Biology (In Press, 1973).

Santisteban, G., and Riley, V.: Thymo-lymphatic organ response to the LDH-virus. Proc. Amer. Assoc. Cancer Res. 14:112 (1973).

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Riley, V.: Erythrocyte sedimentation rate alterations in various malignant diseases as compared with analogous benign pathologies and normal populations. Abstract, 2nd International Symposium on Cancer Detection and Prevention, Bologna, Italy, April 9-12 (1973).

Riley, V.: Characteristics of the lactate dehydrogenase-elevating virus (LDH-virus). In: Slow Virus Diseases, Hotchin, J., ed., S. Karger, Basel (In Press, 1973).

Riley, V.: A device for the automatic scanning, recording, and analysis of density gradient preparations. (Manuscript in Preparation).

Santisteban, G.A., and Riley, V.: Response of the thymo-lymphatic organs and the adrenal cortex to chronic LDH-viremia. (Manuscript in Preparation).

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Riley, V.: Hemodialysis of amino acids: Basic studies *in vitro*. (Manuscript in Preparation).

Riley, V.: A preliminary study of the application of hemodialysis to a lymphoma patient. (Manuscript in Preparation).

Riley, V., and Spackman, D.H.: Theoretical and practical considerations in the use of hemodialysis for the therapeutic removal of asparagine and other amino acids. (Manuscript in Preparation).

Riley, V., Spackman, D.H., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Therapeutic properties of a new glutaminase-asparaginase preparation and the influence of the LDH-virus. (Manuscript in Preparation).

Riley, V., Spackman, D.H., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Alterations in plasma amino acids by a glutaminase-asparaginase preparation and the influence of the LDH-virus. (Manuscript in Preparation).

Memberships in Medical and Scientific Societies:

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12. BIOGRAPHICAL INFORMATION

(Give the following information for all professional personnel listed on page 2, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Santisteban, George Anthony	Senior Biologist	R
PLACE OF BIRTH (City, State, Country)	**PRESENT NATIONALITY** (If non-U.S. citizen, indicate kind of visa and expiration date)	**SEX**
R	R	☒ Male ☐ Female

EDUCATION: *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Montana State University, Missoula, Mon.	B.A. (Honors)	1945	Zoology
University of Utah, Salt Lake City, Utah	M.A.	1949	Zoology
University of Utah, Salt Lake City, Utah	Ph.D.	1951	Anatomy
University of Utah, Salt Lake City, Utah	Postdoctoral	1951-53	

HONORS

American Association for Cancer Research, American Association of Anatomists, Phi Sigma Biological Honorary Society, and New York Academy of Sciences

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Stress, adrenocortical-thymus function and malignancy.	Co-Principal Investigator and Collaborator

RESEARCH SUPPORT *(See instructions)*

$124,089; NIH, USPHS, 09758-05;

Social Stimulation, RNA, and Cardiovascular Disease, 09-01-72 to 08-31-75.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project, including teaching and clinical responsibilities. List all or most representative publications.*

Affiliate, Fred Hutchinson Cancer Research Center, Seattle, Washington, 1973 to present.

Senior Biologist, Pacific Northwest Research Foundation, Seattle, Washington, 1968 to present.

Associate Professor of Biology, Seattle University, Seattle, Washington, 1968 to present.

Acting Head, Department of Biology, Seattle University, Seattle, Washington, 1966 to 1968.

Associate Professor of Biology, Seattle University, Seattle, Washington, 1964 to 1968.

Special Fellowship, U.S.P.H.S. to continue training and do research at the University of Gothenburg (Goteborg), Sweden, under sponsorship of Professor Holger Hyden, 1966 to 1967.

Research Associate at University of Gothenburg, Sweden, to obtain special training in the microanalytical methods for studying intracellular RNA, under sponsorship of Professor Holger Hyden. (On leave from University of Southern California, Research Associate), 1963 to 1964.

Assistant Professor of Physiology (Neurophysiology), University of Southern California School of Medicine, 1959 to 1963.

Assistant Professor of Anatomy (Neuroanatomy, Microscopic Anatomy), University of Southern California, 1954 to 1959.

Assistant Professor (Gross Anatomy), Medical College of Virginia, Richmond, Virginia, 1953 to 1954.

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Postdoctoral Training (Research Instructor) under Dr. Thomas F. Dougherty: Cancer biology, radiobiology, biology of lymphatic tissue and studies of the lymphocytolytic effects of the adrenocortical steroid hormones, 1951 to 1953.

Lecturer in Anatomy (Elementary anatomy, gross anatomy, neuroanatomy, medical histology), University of Utah, College of Medicine, 1949 to 1951.

Teaching Assistant in Anatomy (Elementary anatomy, gross anatomy, neuroanatomy, medical histology), University of Utah, School of Medicine, 1947 to 1949.

Graduate Assistant in Zoology (Comparative vertebrate zoology and mammalian anatomy), Department of Zoology, University of Utah, 1946 to 1947.

Assistant in Zoology (General zoology and comparative vertebrate anatomy), University of Montana, Department of Zoology, 1945 to 1946.

Student Assistant in Zoology, Department of Zoology, University of Montana, 1944 to 1946.

Publications.

Santisteban, G.A.: The growth and involution of lymphatic tissue and its interrelationships to aging and to the growth of the adrenal glands and sex organs in CBA mice. Anat. Rec. 136:1 (1960).

Santisteban, G.A.: The influence of age, sex, and the post-operative time interval upon the growth of lymphatic tissue following adrenalectomy in CBA mice. Anat. Rec. 137:4 (1960).

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Santisteban, G.A.: The response of the thymolymphatic system to graded doses of ethyl alcohol and its relationship to adrenocortical activity. Quart. J. Stud. Alc. 22:1 (1961).

Santisteban, G.A.: Adrenocortical influences upon the reconstitution of lymphatic tissue following acute involution. Endocrinology 64:5 (1959).

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Santisteban, G.A., Riley, V., and Willhight, K.: Studies in virus-tumor relationships: Responses of the adrenocortical-thymolymphatic systems to the LDH-elevating virus. Abstracts, Tenth International Cancer Congress, p. 302 (1970).

Santisteban, G.A., Gustander, C., and Willhight, K.: Studies of the maturation of the adrenal cortical-lymphatic tissue interrelationships. Anat. Rec. 163:2 (1969).

Santisteban, G.A.: Studies of the development of adrenal cortical function. Proceedings, IX International Congress of the Anatomists, Leningrad (1970).

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Henry, J.P., and Santisteban, G.A.: The induction of arteriosclerosis by psychosocial factors in CBA mice: Observations on the heart, aorta, and kidneys. Atherosclerosis 14:203-218 (1971).

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Santisteban, G.A.: The thymus involutionary and adrenal cortical responses to stress in normal, isolation-reared, and socially aggregated CBA mice of 8, 15, and 52 weeks of age, and their relation to renal and cardio-vascular disease. Anat. Rec. (In Press).

Fitzmaurice, M.A., Riley, V., and Santisteban, G.A.: Biological synergism between the LDH-virus and *Eperythrozoon coccoides*: Studies on the mechanism. Path.-Biol. 20:743-750 (1972).

Santisteban, G., and Riley, V.: Thymo-lymphatic organ response to the LDH-virus. Proc. Amer. Assoc. Cancer Res. 14:112 (1973).

Santisteban, G.A., and Riley, V.: Response of the thymo-lymphatic organs and the adrenal cortex to chronic LDH-viremia. (Manuscript Submitted).

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12. BIOGRAPHICAL INFORMATION

(Give the following information for all professional personnel listed on page 2, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
SPACKMAN, Darrel H.	Senior Research Biochemist	R

PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
R	R	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Utah, Salt Lake City, Utah	B.A.	1950	Chemistry
University of Utah, Salt Lake City, Utah	M.A.	1952	Biochemistry
University of Utah, Salt Lake City, Utah	Ph.D.	1954	Biochemistry

HONORS
Phi Betta Kappa
American Society of Biological Chemists
American Association for Cancer Research

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Amino acids, peptides and proteins involved in malignancy	Co-Principal Investigator

RESEARCH SUPPORT *(See Instructions)*

Supported by grants awarded to Dr. Vernon Riley as Principal Investigator

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project, including teaching and clinical responsibilities. List all or most representative publications.*

Senior Research Biochemist, Department of Microbiology, Pacific Northwest Research Foundation, Seattle, Wash., 1968 to present.

Research Assistant Professor of Biochemistry and of Obstetrics-Gynecology, University of Washington, Seattle, Wash., 1963 to 1968.

Senior Biochemist, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif. 1959 to 1963.

Research Associate, Rockefeller Institute, New York, N.Y., 1954 to 1959.

Postgraduate Fellowship, U.S. Public Health Service, 1951 to 1954.

Publications

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Smith, E.L., Davis, N.C., Adams, E., and Spackman, D.H.: The specificity and mode of action of two metal peptidases. In: The Mechanism of Enzyme Action, McElroy, W.D., and Glass, B., eds., Johns Hopkins Press, Baltimore, pp. 291-312 (1954).

Spackman, D.H., Smith, E.L., and Brown, D.M.: Leucine aminopeptidase IV. Isolation and properties of the enzyme from swine kidney. J. Biol. Chem. 212:255 (1955).

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Stein, W.H., Kinkel, M.G., Cole, R.D., Spackman, D.H., and Moore, S.: Observations on the amino acid composition of human hemoglobins. Biochim. Biophys. Acta., 24:640 (1957).

Moore, S., Spackman, D.H., and Stein, W.H.: Chromatography of amino acids on sulfonated polystrene resins. An improved system. Analyt. Chem. 30:1185 (1958

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Spackman, D.H., Stein, W.H., and Moore, S.: The disulfide bonds of ribonuclease. J. Biol. Chem. 235:648 (1960).

Spackman, D.H.: "Instruction Manual and Handbook, Model 120 Amino Acid Analyzer," for Spinco Division, Beckman Instruments, Inc., Copyright, 1960.

Spackman, D.H.: Accelerated system for the automatic analysis of amino acids. Fed. Proc. 22:224 (1963) (Abstract).

Spackman, D.H.: Ion exchange chromatography of protein hydrolysates. A. The automatic amino acid analyzer and accelerated procedures. B. Method for measurement of amino acids in proteins by ion exchange chromatography on the amino acid analyzer. In: Serum Proteins and the Dysproteinemias, Sunderman, F.W., and Sunderman, F.W., Jr., ed., Lippincott, Philadelphia, pp. 166-184 (1964).

Spackman, D.H.: The free amino acids in human amniotic fluid. Fed. Proc. 25:798 (1966).

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Spackman, D.H.: Proteins: Amino acid analysis and content. In: Encyclopedia of Biochemistry, Williams, R.J., and Lansford, E.L., Jr., eds., Reinhold, New York, pp. 685-687 (1967).

Spackman, D.H.: Section I. Amino acid analysis and related procedures. 1. Accelerated methods. In: Methods in Enzymology, Hirs, C.H.W., ed., Academic Press, New York, Vol. XI, pp. 3-15 (1967).

Spackman, D.H.: Improved resolution in amino acid analysis in cancer therapy studies. Fed. Proc. 28:898 (1969) (Abstract).

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Riley, V., Spackman, D., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Studies on a new glutaminase/asparaginase: Therapeutic properties, half-life, and alterations in the plasma amino acids. Proc. Amer. Assoc. Cancer Res. 13:117 (1972) (Abstract).

Spackman, D., and Riley, V.: Studies in Leukemia: The levels of asparagine, glutamine, asparaginase, and glutaminase in the mouse following asparaginase administration. Proc. Amer. Assoc. Cancer Res. 13:81 (1972) (Abstract).

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Spackman, D., and Riley, V.: Immunologically-altered response to asparaginase in the mouse: Influence of the LDH-virus. Proc. Amer. Assoc. Cancer Res. 14:113 (1973) (Abstract).

Riley, V., and Spackman, D.: Amino acid profiles in melanoma tissues and host plasmas. Yale Journal of Biology 1973 (In Press).

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Riley, V., Spackman, D.H., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Therapeutic properties of a new glutaminase-asparaginase preparation and the influence of the LDH-virus. Cancer Res. 34:429-438 (1974).

In addition, the following manuscripts have been submitted or are being prepared and will be submitted for publication in the near future:

Riley, V., and Spackman, D.H.: Theoretical and practical considerations in the use of hemodialysis for the therapeutic removal of asparagine and other amino acids. (Manuscript Submitted).

Riley, V., Spackman, D.H., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Alterations in plasma amino acids by a glutaminase-asparaginase preparation and the influence of the LDH-virus. (Manuscript in Preparation).

Riley, V., Spackman, D., and Fitzmaurice, M.A.: Asparaginase: An experimental and clinical model for new concepts in cancer research. CRC Critical Reviews. (Manuscript in Preparation).

Spackman, D.H.: Amino acid analysis of physiological samples with a high resolution, high sensitivity system. (Manuscript in Preparation).

Spackman, D.H., and Riley, V.: Free amino acids in the plasma, urine, and tissues of mice: Effects of sample preparation, fasting, and tumor growth. (Manuscript in Preparation).

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

R: REDACTED MATERIAL

14. PROVISIONAL BUDGET FOR THE FIRST 12-MONTH PERIOD

SALARIES		Salary	F/B	Total
Dr. Vernon Riley	Co-Principal Investigator			
Dr. George Santisteban	Co-Principal Investigator			
Dr. Daniel Spackman	Co-Principal Investigator			
D. Lucille Bredberg	Research Associate	REDACTED		REDACTED
To be hired	Research Assistant			
	Total			

Note: All salaried personnel will contribute 100% of their time to this project. The non-salaried co-principal investigators will contribute their time as required by the project.

CONSUMABLE SUPPLIES

Mice, food, bedding, disposable syringes, needles bleeding tubes, miscellaneous glassware, reagents, special supplies for the amino acid analyzer and the Coulter Counter, and the florescent spectrophotometer, miscellaneous supplies and minor items of equipment. $ 6,000

SPECIAL SERVICES

Medical illustrations, photography, slide preparations, reprints, page charges, duplication, equipment maintenance. $ 2,000

SPECIAL EQUIPMENT

Instrument for measuring blood pressure in mice. (Tail plethysmography: pressure cuff, strain gauge, Sanborn recorder.) Approx. $ 5,000

TOTAL $55,979

INDIRECT COSTS: To be negotiated.

15. ESTIMATED TIME REQUIRED TO COMPLETE THESE STUDIES:

It is obvious that all of the various experimental ramifications of these studies cannot be completed during the 12-month period of this budget. It is suggested that initial approval for a two year period be considered, with the amount for continuing support being determined by the rate at which the council feels the studies justify at the end of the first year. No special equipment is anticipated for the 2nd year.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

May 21, 1974

MEMORANDUM

TO: RCH, JHK, FWN

FROM: WUG

SUBJECT: Tests for Stress.

On May 14 and 15, Vernon Reilly of Pacific Northwest Research Center, visited CTR. He spoke about some studies that his associates had been doing on manifestations of stress. In a remote way they were a continuation of some studies of a former associate of mine. Reilly has for many years worked with lactic acid dehydrogenase, an enzyme that is produced by a virus that is carried by many transplanted tumors. The virus is also carried in mice and is apparently slightly stressful. Reilly has also developed a caging system that puts a minimum of stress on his mice.

Experiment 1. C3H mice. Group 1 with LDH in regular cages. Group 2 in regular cages. Group 3 in the stressless cages.

Results: Group 1 - 90%+ mammary cancers by 70 weeks.
Group 2 - 70%+ mammary cancers by 70 weeks.
Group 3 - 0% mammary cancers by 50 weeks when about 70% and 50% of Groups 1 & 2 respectively had cancers.
All mice had the mammary tumor agent.

Experiment 2. Mice produce reportedly corticosterome (cs) exclusively as the adrenal glucocorticoid. A Dr. Spakman(?) at PNR has developed a sensitive method of quantitating cs in 0.05 ml plasma. They found that when mice arrived in their laboratory the cs was always high but declined, reaching base line levels in 2 weeks. Interpretation: shipping of mice is stressful.

Observation 3. Blood for the cs determinations is drawn from the retro-orbital veins. If one routinely bleeds a cage of mice he obtains very erratic levels of cs. If one takes animals 1 to 10 all in the same cage, removing 1 mouse every 3 or 4 minutes, the cs levels in 1 will be low and there will be a progressive increase in the cs levels up to mouse 10. It takes 3 or 4 minutes for the stress to be manifest and it is additive. Actually, if 2 cages are side by side the mice in the second cage will be influenced.

Observation 4. Mice were stressed and their cs starts to rise within a few minutes and stays up for 3-6 hours. (There are diurnal rhythms.) Lagging about one hour, as I recall, the circulating T lymphocytes almost entirely disappear as indicated by the decreased lymphocyte counts. (I did not go into the way he determined they were T lymphocytes. They were determined by plaque formation on

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

SRBC so apparently hemolytic. The thymus starts to decrease in size. There is a decrease in the number of plaque-forming lymphocytes as determined by lysis of sheep red blood cells. Stress determinations can be done in 1 hour by cs, a bit later by the lymphocyte counts, and later by the plaque method. Presumably all are measurements of a response to stress.

Reilly did not have data on the influence of the repetition of stress or adaptation. He had no data on the strain differences in the response to stress. He did know that the responses were greater at one part of the day than at another.

Some thoughts: Might it not be a good idea to compare the stress responses of the strains that are to be used on the smoking experiment? What is the relation of the AHH inducibility to stress responses? Corticoids are great inducers of some hepatic enzyme systems. Tyrosince transaminase is, I believe, one of them. What do the goat-cell plaque counts being done at Scripps show? If there are differences, could they be associated with cs levels? With diurnal rhythms? Much of this work has not been published and there is need for some confidentiality.

W.U.G.

WUG:ek

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#814A - RYAN

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 5, 1974

Grant application No. 814A

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Liebow, Sommers

Subject: Una S. Ryan, Ph.D., Papanicolaou Cancer Research Institute, Miami, Florida
Continuation application No. 814A
"Endocrine Functions of the Lungs"

History

CTR Grant No. 814 "The Role of Endothelial and Epithelial Cells in Non-Ventilatory Functions of the Lungs", with renewals, is now completing a three year program approved for 1972 through 1974.

Although the enclosed has a new title, the proposal essentially is a continuation of the same study.

Request

Application No. 814A requests $37,902 plus two additional years. As a "continuation" it competes on the same basis as a new application.

Documents submitted (attached)

1. Application 7/30/74, with C.V.'s of Drs. U. S. Ryan and J. W. Ryan.

2. Reprint of the paper checked on Page 9-13 of the application.

F.W.N.

FWN:wg
Encls.

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#914A

THE COUNCIL FOR TOBACCO RESEARCH, U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED AUG 5 1974

Application for Research Grant
(Use extra pages as needed)

Date: 7/30/74

1. Principal Investigator (give title and degrees):

Una S. Ryan, Ph.D., Senior Scientist
Papanicolaou Cancer Research Institute
Assistant Professor of Medicine, University of Miami School of Medicine

2. Institution & address:

Papanicolaou Cancer Research Institute
1155 N.W. 14th Street
Miami, Florida 33136

Mailing Address: P.O. Box 6188
Miami, Fla. 33123

3. Department(s) where research will be done or collaboration provided:

Cardiopulmonary Unit
1425 N.W. 10th Avenue
Miami, Florida 33136

4. Short title of study:

ENDOCRINE FUNCTIONS OF THE LUNGS

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

There are two main aims of this research proposal: the first is to obtain direct evidence on the precise subcellular site of pulmonary angiotensin converting enzyme and to relate these data to understanding of the non-ventilatory functions of the lung. The second aim is to develop rapid, simple and sensitive assays of pulmonary angiotensin converting enzyme to facilitate future studies of the effects of physiologic changes and environmental influences (e.g. tobacco smoke) on the ability of the lungs to convert angiotensin I to its potent lower homolog, angiotensin II.

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B. Brief statement of working hypothesis:

A. It is now well recognized that the lungs process the circulating vasoactive polypeptides, bradykinin and angiotensin I, the former being inactivated while the latter is activated. Previously, we have postulated that the enzymes responsible for the metabolism of these polypeptides are situated on or close to the luminal surface of pulmonary capillary endothelial cells. Studies from our laboratory over the past six years, as well as from others, have supported the hypothesis but have not proved it. Within the last year, it has been shown that the enzyme that activates angiotensin I (by conversion to angiotensin II) is also responsible for the inactivation of bradykinin. We have raised antibodies to this enzyme and now propose to use the antibodies, labelled for immunocytochemistry, to provide a definitive test of our hypothesis.

B. Our previous studies on the metabolism of the vasoactive polypeptide hormones required intrinsically labelled synthetic hormones. The hormones and their radioactive metabolites were identified after lung perfusion by chromatographic, electrophoretic and countercurrent distribution techniques requiring long periods of time. Whereas this approach was essential for defining the biochemical mechanisms by which the hormones were metabolized, it is evident that this approach does not yield itself to studies of instantaneous changes in pulmonary function that may occur as a result of subtle influences such as tobacco smoking. Therefore, in addition to testing the hypothesis described above, we would like to undertake a technical program to develop inexpensive, radioactive substrates which will lend themselves to the rapid, simple and sensitive measurement of the turnover rate of angiotensin converting enzyme. Such substrates will be of immediate use in investigations of the ways in which the lungs can modulate their "endocrine" functions in response to physiological stimuli and to inhalants such as tobacco smoke.

9. Details of experimental design and procedures (append extra pages as necessary)

Appended. See section beginning 9-1.

10035443 19

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The investigators of this program constitute the Cardiopulmonary Unit of the Papanicolaou Cancer Research Institute. These laboratories occupy the Sieron Building, situated within the University of Miami Medical Center. Our laboratories include about 5,000 square feet of floor space, and include a cold room, a secretarial area, vivarium, and rooms for electron microscopy, biochemistry and polypeptide synthesis.

Our laboratories have access to the full facilities of the Papanicolaou Institute and of the Department of Medicine, University of Miami. We also have free access to the full library facilities of the University of Miami Medical Center.

Equipment

Philips EM 301 electron microscope
LKB Ultrotome III
LKB knife maker
Durst enlarger and ancillary photographic equipment
Zeiss binocular microscope with camera and phase contrast attachments
Wild dissecting microscope
Gilson and LKB fraction collectors
Craig-Post 200-transfer countercurrent
Spinco 120C amino acid analyzer
Unilux II liquid scintillation counter
Actigraph strip counter
Buchi flash evaporator
Two-channel autoanalyzer
VirTis lyophilizer
Apparatus for manual and automatic synthesis of polypeptides
Circulating water baths and incubators
Pumps for infusion and respiration of small animals
Apparatus for thin-layer chromatography and electrophoresis
Mettler top pan balance
Radiometer pH meters (2)
Radiometer automatic titrator
Grass four-channel polygraph

11. Additional facilities required:

No new facilities are required for the purposes of this grant.

12. Biographical sketches of investigator(s) and other professional personnel (append):

Appended

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Appended

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
*Una Ryan, Ph.D.	Principal Investigator	
**J.W. Ryan, M.D., D.Phil.	Co-Investigator	
**A. Chiu, Ph.D.	Co-Investigator	
Fringe Benefits		REDACTED
Technical		
Debra Hodges	Lab Tech	REDACTED
Erica Clements	Lab Tech	
Sub-Total for A		26,383
B. Consumable supplies (by major categories)		
Animals: rats $400, rabbits, $200, Golden hamsters, $100		700
Photographic supplies		1,000
Chemicals and glassware, general		800
Fixatives, embedding and staining materials		700
Microperoxidase, 250 mg		600
Reagents for peptide synthesis and conjugation techniques		700
Sub-Total for B		4,500
C. Other expenses (itemize)		
Travel (1 trip per investigator @ $500 each)		1,500
Shipping charges		150
Reference Books		75
Regrinding diamond knives for microtomy		350
Sub-Total for C		2,075
Running Total of A + B + C		32,958
D. Permanent equipment (itemize)		-0-
Sub-Total for D		-0-
E. Indirect costs (15% of A+B+C)		4,944
Total request		37,902

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	29,021	4,770	2,075	-0-	5,380	41,246
Year 3	31,923	5,056	2,075	-0-	5,858	44,912

10035443 21

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BUDGET JUSTIFICATION

*Una Ryan. The major portion of Dr. Una Ryan's salary is paid by the American Heart Association through her tenure of an Established Investigatorship. The salary requested here is calculated as 10% of the allowed supplement.

**James W. Ryan and Andrew Chiu. Salary funds are requested in proportion to per cent times to be spent on this research project. Dr. Chiu's biographical sketch is attached.

Fringe benefits are calculated at 15% of salaries and include social security, health insurance, life insurance, unemployment and pension plan.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Endothelium: Structure and Functions (salary only)	American Heart Association (#72 160)	16,000	7/1/74 - 6/30/75
*Fine Structure of Normal Lung Cells in Situ	National Heart and Lung Institute (#NO1-HR3-3015)	45,991	10/1/74 - 9/30/75

*The work solicited by the contract of the National Heart and Lung Institute does not overlap in approach, concept or timing with the research program described in the present application.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Una S. Ryan

Signature Una S. Ryan Date 8/1/74

Telephone 305 (Area Code) 324-4341 (Number) (Extension)

Checks payable to

Papanicolaou Cancer Research Institute

Mailing address for checks

P.O. Box 6188

Miami, Florida 33123

Responsible officer of institution

Typed Name Dr. Julius Schultz

Title Director and President

Signature [signature] Date 8/2/74

Telephone 305 (Area Code) 324-5572 (Number) 34 (Extension)

10035443 23

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9. Details of experimental design and procedures

1. Final approach to enzyme localization by immunocytochemistry

Background

Over the past six years, a large body of indirect evidence has been presented indicating that a variety of polypeptide hormones, prostaglandins, nucleotides and amines disappear during circulation through the lungs (see Vane, 1969, for review). Some six years ago, we began a series of experiments to examine the mechanisms by which these phenomena occur. In brief, evidence at hand indicates that circulating adenine nucleotides, angiotensin I and some of the kinins are metabolized by hydrolase enzymes on or near the luminal surface of pulmonary endothelial cells (Ryan et al., 1968, 1970a and b, 1971, 1972; Ryan and Smith, 1971a and b, 1973; Smith and Ryan, 1969, 1970, 1971 a and b, 1972, 1973). By contrast, prostaglandins of the E and F series appear to be removed from the circulation by cellular uptake.

From the beginning of our experiments, it was evident that we had found an entirely new mechanism of hormone metabolism (Ryan et al., 1968). Firstly, blood enzymes are not required. Secondly, the lungs do not secrete hydrolase enzymes having the specificity required to degrade angiotensin I, bradykinin or adenine nucleotides. Thirdly, lungs perfused with radioactive angiotensin I, bradykinin or nucleotides do not retain radioactivity. On these points alone, we began to suspect that the requisite hydrolase enzymes exist on or very near the luminal surface of pulmonary endothelial cells. The concept was further supported by examination of the kinetics of disappearance of intrinsically-labelled parent compounds. We found that the mean transit times and volumes of distribution of radioactivities are no greater than those of blue dextran (MW > 2,000,000), a substance unlikely to leave the vascular space. Using radioactive bradykinin, adenine nucleotides or angiotensin I, there is a quantitative recovery of radioactivity in the pulmonary venous effluent; yet little, if any of the radioactivity remains in the form of the parent compound.

In parallel studies, we began investigations of the morphology of pulmonary endothelial cells, placing special emphasis on the occurrence of fine structure of the plasmalemma that might explain the phenomena described above. It was evident in the first studies that pulmonary endothelial cells, especially those of capillaries, are densely packed with caveolae (pinocytotic vesicles), most of which communicate with the exterior of the cell through small stomata: It was also evident that the caveolae along the luminal surface communicate directly with circulating blood and have the effects of expanding the surface area of the cell itself and of increasing the volume of the vascular lumen (Smith and Ryan, 1970).

Endothelial caveolae became of even greater interest as a consequence of our finding that the caveolae contain ATPase and 5'-nucleotidase, enzymes apparently not shared by the remainder of the plasma membrane (Smith and Ryan, 1970, 1971). Recently, we have shown by combined biochemical and cytochemical techniques that 5'-AMP entering the pulmonary artery is, in fact, dephosphorylated by enzymes of the caveolae open to the vascular lumen (Ryan and Smith, 1971b).

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

Whereas the data on the endothelial processing of adenine nucleotides is direct, evidence on the processing of angiotensin I and bradykinin is still, to an extent, inferential. Specific cytochemical tests are not available for polypeptide hormones. Nonetheless, our hypothesis on the means of metabolism of angiotensin I and bradykinin has been tested by a variety of techniques. Firstly, one implication of our hypothesis is that angiotensin I and bradykinin should be metabolized to characteristic products by the plasma membrane fraction of whole lung homogenates. We have found that the lung plasma membrane fraction does, in fact, convert angiotensin I to angiotensin II and reduces ^{14}C-Pro^{2}-bradykinin to radioactive Prolyl-Proline (Ryan and Smith, 1971a). Secondly, isolated pulmonary endothelial cells should, on incubation with the parent peptides, produce the same products. This has been found to be the case (Ryan and Smith, 1973; Smith and Ryan, 1973).

Having adduced evidence that the membranes of endothelial caveolae have metabolic properties apparently not shared by the remainder of the plasmalemma, we have begun to examine caveolae for ultrastructural specializations. Data obtained so far indicate that caveolae have a circular skeletal structure, possibly functioning to maintain the patency of the stomata and the integrity of the stomal diaphragm (Smith and Ryan, 1972). In studies of thin sections, it has also become apparent that the inner, concave surface of the caveola membrane contains regularly-spaced globular structures, possibly enzymes or binding sites (Smith and Ryan, 1972, 1973; Smith et al., 1973). Our freeze-fracture studies have confirmed and extended these findings. Of particular interest, intramembranous particles (75-100 Å in diameter) are prominent in association with caveolae. The size of these particles, some of which are thought to be enzymes, is about that expected for a globular enzyme having a molecular weight of 150,000-300,000. This point may be in agreement with the findings of Dorer et al. (1972), which show that the angiotensin I converting enzyme of hog lung has a molecular weight of 280,000. Our recent experience with negatively-stained converting enzyme shows the enzyme to have a diameter of about 60 Å. Taking into account the fact that the platinum-carbon shadowing material used to produce freeze-fracture replicas is of finite thickness (about 20 Å), it appears reasonable that replicas of converting enzyme itself would approach 100 Å.

Having been supplied with a homogeneous preparation of hog lung converting enzyme (Dorer et al., 1972), we now have the possibility of localizing the subcellular site of the pulmonary conversion of angiotensin I to angiotensin II by immunocytochemical means.

Immunization

We have immunized goats with hog lung converting enzyme using complete Freund's adjuvant. We have on hand liter quantities of an antibody, which by all techniques tested appears to be specific in its reaction with converting enzyme. The antibody has been examined by double immunodiffusion, immunoelectrophoresis and for inhibitory effects on converting enzyme activity.

Graph 1 shows the inhibitory effects of increasing amounts of whole antiserum. The goat which produced this antiserum has been boosted twice more since we obtained these data.

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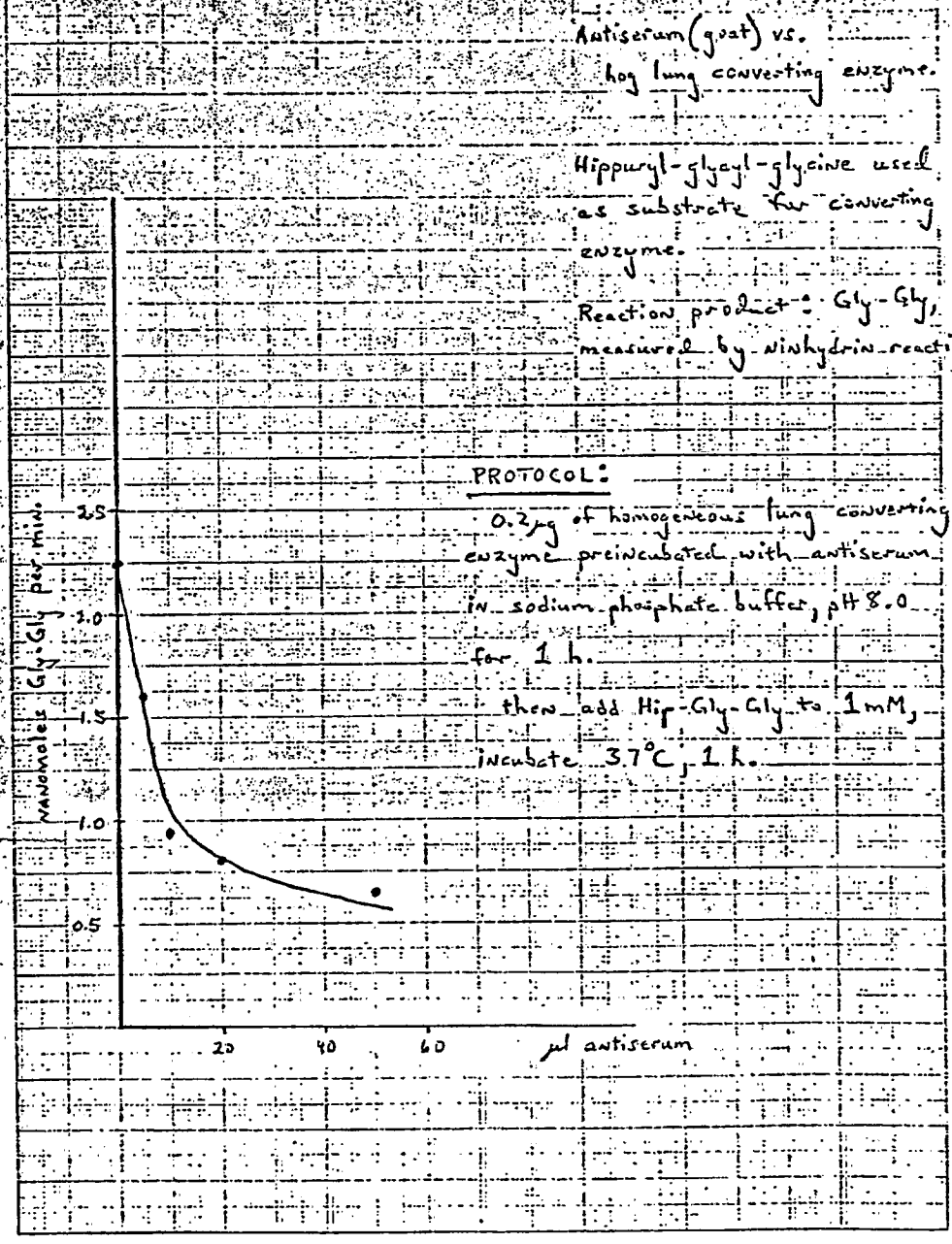
GRAPH 1 9-3

INHIBITION OF CONVERTING ENZYME



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GRAPH 2 9-4

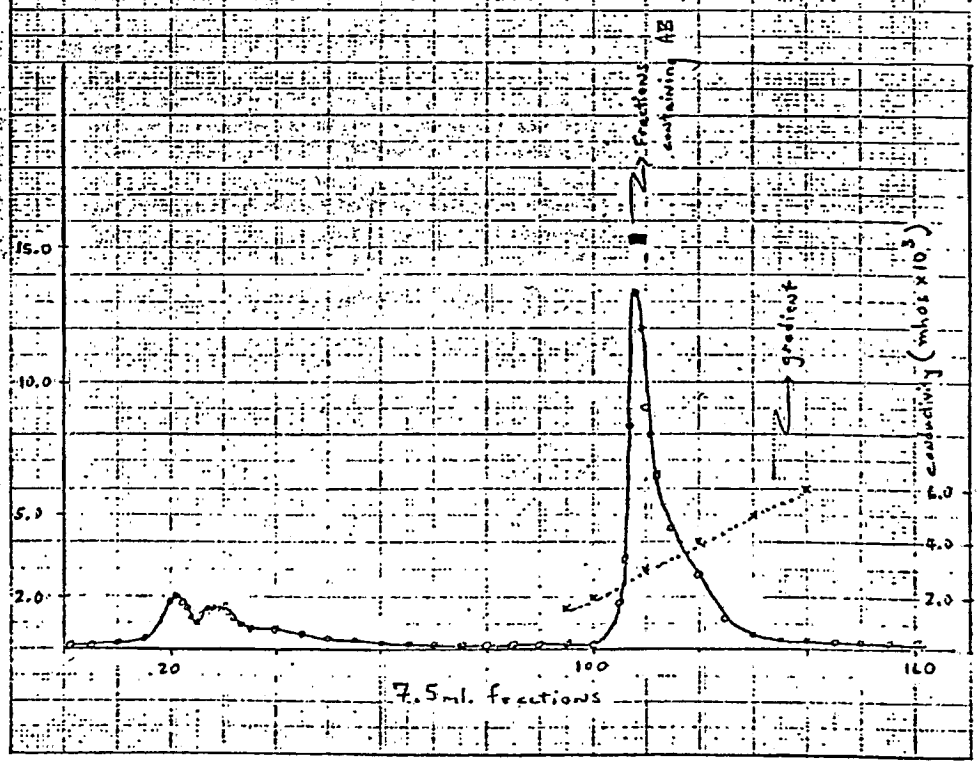
PURIFICATION OF ANTIBODY TO HOG LUNG CONVERTING ENZYME

① Goat antiserum (50 ml) was treated with $(NH_4)_2SO_4$, 33% final.

② The precipitate was washed with 33% $(NH_4)_2SO_4$ and then was dissolved in 0.15 NaCl (50 ml).

③ The saline-AB solution was dialyzed vs. 5mM sodium phosphate - 10mM NaCl, pH 7.5 for 48 h.

④ The dialyzed material was applied to a column containing DEAE-cellulose (3 x 25.2 cm; 0.005M sodium phosphate - 0.01M NaCl, pH 7.5; flow rate 20 ml/h). A linear gradient was developed to 0.3M NaCl.



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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

Purification of antibody

Much of the time and effort leading up to the localization of converting enzyme by immunocytochemical techniques has focused on the purification of the antibody to eliminate non-specific reactants. The purification procedures are outlined on graph 2. The antibody was precipitated with ammonium sulphate and then chromatographed on DEAE-cellulose using a linear salt gradient. Normal goat serum, for control studies, was processed similarly.

Conjugation of antibody to marker

We chose to couple antibody to microperoxidase for pilot experiments. Microperoxidase has a major advantage over tracers such as ferritin and horseradish peroxidase in that its monomeric structure is small (MW approx. 2,000). Thus, antibody conjugated to microperoxidase can be readily separated from unconjugated microperoxidase and even from small polymers of microperoxidase that may occur as side products of the conjugation technique.

Pilot experiments

We have coupled microperoxidase to our purified antibody by glutaraldehyde (Avrameas, 1969, 1970). The conjugate was dialyzed and the non-dialyzable portion was used for cytochemical studies.

Our experiments were modeled on the published techniques of Moriarty and Halmi (1971), Mazurkiewicz and Nakane (1972), and Graham and Karnovsky (1966). We have made progressive modifications, and procedures as they are now conducted in our laboratory are detailed below.

A rat is prepared for perfusion of the lungs as described previously (Smith and Ryan, 1970). The lungs are pumped with Krebs-Henseleit solution until they are freed of blood and then perfused for 10 min with paraformaldehyde-picric acid as fixative (Stefanini et al., 1967).

The paraformaldehyde-picric acid fixed blocks are diced extremely finely under the binocular microscope and then incubated for 30 min in each of the following mixtures. Phosphate buffered saline (PBS) is used as a wash. All incubations are carried out at room temperature on a shaker. All solutions are prepared immediately before use.

1) Normal goat serum 1:30 (to cover non-specific sites)
   Wash in PBS

2) Converting enzyme Ab/microperoxidase complex, at a dilution of 1:100, but also at dilutions of 1:50 or 1:25

3) Diaminobenzidine (DAB)

4) H_2O_2 added to DAB for final 1 min

5) OsO_4 (1% in veronal acetate buffer) 1 hr
   Dehydration in graded ethanol series
   Embedment in Araldite via propylene oxide

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

We have obtained sections which show no impairment of fine structural preservation. The sections are clear and show a fine and precise localization of microperoxidase.

The reaction product is present on much of the luminal plasma membrane but is most prominent on the membranes of caveolae. One striking finding was that endothelium of small vessels, probably small venules, showed a marked localization particularly on the endothelial projections. During the course of our previous ultrastructural studies of pulmonary endothelial cells, we had occasion to examine the luminal surface of the pulmonary artery by scanning electron microscopy. We found that the luminal surface is not flat but is covered by a profuse, irregular array of minute projections (Smith et al., 1971). The function of the projections is not known but it is clear that they vastly increase the surface area of the endothelium and cannot fail to affect flow dynamics at the cell body. Their size and density would produce a meshwork allowing an eddy flow of cell-free plasma over the main body of the cell and we suggested they might have implications in the exchange of metabolites between endothelium and blood. In view of the localization of converting enzyme activity on endothelial projections, we feel it will be useful to determine their role in the metabolism of circulating substances. The prominence of reaction product on projections of venules suggests the possibility that, e.g. the conversion of angiotensin I to angiotensin II occurs immediately after central venous blood is converted to arterial blood. We plan to focus attention on this point.

While it is clear that these pilot experiments require much refinement they serve to show the feasiblity of the technique.

Conjugation of angiotensin converting enzyme to 8-MP

Our pilot studies have used techniques that might be termed "state of the art." We have come to recognize, as have others, that the "state of the art" techniques require improvement. The major problem centers around the methods of conjugating specific antibody to a marker, e.g. microperoxidase, such that the immunoreactivity and specificity of the antibody are not impaired and the activity of the enzyme is left intact. In fact, the commonly used conjugation techniques, such as those employing glutaraldehyde or carbodiimide, result in the formation of a large number of side products, virtually all of which have the potential of causing artifactual results. For example, in addition to the formation of antibody-marker hybrids, it is likely that antibody-antibody, marker-marker and other complex (straight chain and branched) polymers are formed. Of the desired antibody-marker hybrids, one cannot be sure that each such complex retains immunoreactivity and enzyme activity. The active antibody-inactive enzyme species can be expected to bind antigen, but in a manner so as to be undetectable by electron microscope techniques. The inactive antibody-active enzyme species may yield non-specific electron-dense deposits. In addition, the possibility must be considered that some antibody and some marker molecules do not conjugate but remain in their original states.

Within limits, undesired side products of the conjugation techniques can be eliminated or reduced by separation procedures, e.g. gel filtration.

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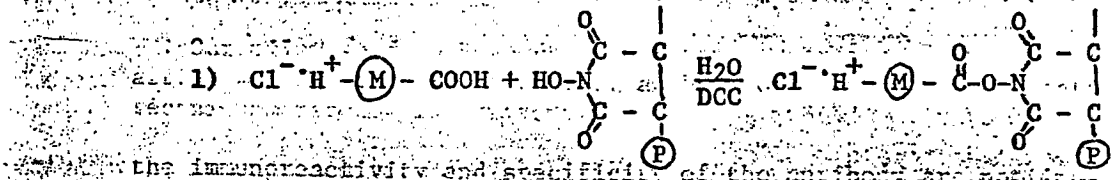
Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

When immunoabsorption is possible, one can purify active antibody-marker hybrids. However, immunoabsorption is feasible only when antigen is in sufficient supply. Unfortunately, pure angiotensin converting enzyme is in extremely short supply. In any event, immunoabsorption is selective for antibody and not for marker and therefore does not separate active antibody-active enzyme from active antibody-inactive enzyme hybrids, and would not, as a single procedure, eliminate unreacted antibody.

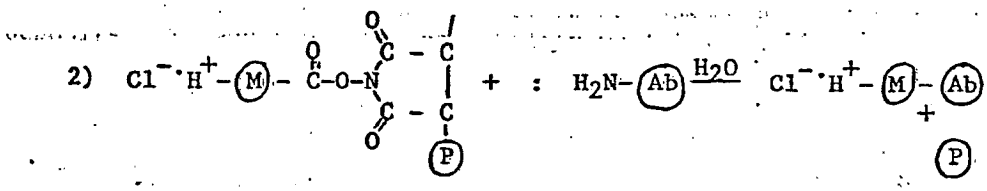
Thus we propose to undertake a program to develop improved, more specific methods of conjugating antibody to microperoxidase (11-MP) or to its lower homolog, the heme-octapeptide (8-MP). It should be emphasized that success using antibody to angiotensin converting enzyme will be immediately translatable for use with other antibodies, including antibodies to nicotine and cotinine. Antibodies to the latter compounds are under development in a parallel program of this laboratory (see Appendix).

We project two general approaches for improving conjugation techniques. In the first approach, we plan to use a technique developed by a member of this laboratory for solid phase polypeptide synthesis (Laufer, 1968). The marker, 11-MP or 8-MP will be covalently bound to an insoluble N-hydroxy-succinimide polymer (copoly-ethylene-N-hydroxy maleimide).

Legend - P = polymer
M = marker
AB = antibody



Reaction 1) employs the marker in its hydrochloride form to prevent cyclization. The DCC may lead to polymerization of some of the marker, but these markers can be separated from the derivatized insoluble polymer by filtration. In addition, the hydrochloride will tend to limit polymerization. The desired reaction of marker to the polymer will yield an active ester linkage.



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Reaction 2) effects the conjugation, and leaves the marker-antibody molecule in solution and thus readily separable from the polymer. The only other reactants to be dealt with should be unreacted antibody. The [illegible] concentration of unreacted antibody can be minimized by using the polymer in excess.

The only major problem that we foresee at this time is possible steric hindrance. The antibody constitutes an extremely bulky molecule in comparison with the functional groups of the polymer. Should the problem of steric hindrance arise, we will attempt to develop an alternative scheme using Fab fragments and/or conjugation "bridges" (see below).

Our second appraoch represents a contingency plan: Jamieson and colleagues of the Rockefeller University (Kraehenbuhl et al., 1974) have developed an elegant conjugation scheme but of such complexity that few laboratories could undertake it. In brief, Jamieson's group isolated microperoxidase from the peptic digestion of cytochrome C. The heme-undecapeptide (11-MP) was then reacted with trypsin to remove the N-terminal tripeptide, Val-Gln-Lys. The resulting heme-octapeptide (8-MP) was then isolated from the other reactants by an extensive countercurrent distribution involving over 2,000 transfers. The 8-MP was derivatized after synthesis and purification of the N-hydroxysuccinimide ester of p-formylbenzoic acid. The ester was reacted with the α-amino group in the presence of pyridine. The activated 8-MP was reacted with antibody to form a Schiff base and the aldehyde group of the derivatized 8-MP was reduced with borohydride. Unreacted 8-MP and unconjugated antibody were removed by gel filtration and ion exchange chromatography (DEAE-cellulose).

Our laboratory is fully staffed and equipped to carry out the techniques described by Jamieson and colleagues. However, we believe it feasible to simplify their techniques, hopefully to a point such that specific conjugations can be carried out by a larger number of laboratories. One development which should make our job easier is that microperoxidase (11-MP) is now available commercially (Sigma). This eliminates the steps required for the isolation of cytochrome C, its degradation with pepsin and isolation of 11-MP itself. Secondly, although we have countercurrent distribution apparatus, we feel that we can simplify the separation of 11-MP from 8-MP by high-voltage electrophoresis, 11-MP being more positively charged because of its lysyl residue. An electrophoretic separation could be effected in a matter of one or two hours versus the week or more required for counter-current distribution of greater than 2,000 transfers.

Should our first approach (using the insoluble polymer of N-hydroxy-succinimide) work, it will not be necessary to pursue the Jamieson approach. However, should the problem of steric hindrance to reaction of antibody with the polymer prove insuperable, we can combine favorable features of the first approach with those of the second approach; which is to say that we can form an active ester of polymer and p-formylbenzoic acid and then react 8-MP to form the activated 8-MP of the Jamieson procedure. The activation of 8-MP by this scheme should occur spontaneously. By using p-formylbenzoic acid in reaction with the N-hydroxysuccinimide polymer it will not be necessary to isolate the active ester as is necessary in the Jamieson procedure. As in the Jamieson procedure, the p-formylbenzoic acid moiety will "bridge" the antibody to its marker. The remainder of the conjugation to antibody could proceed as described by Jamieson and colleagues.

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The coupling of angiotensin converting enzyme antibody to 8-MP should be of great advantage in our EM localization study. Because of its smaller size (<15 Å diameter), it should result in less alteration of the biological activity of ligands to which it is coupled. In addition, the small size is particularly important for immunocytochemical techniques in which the tagged antibodies are required to penetrate several diffusion barriers such as the plasma membrane and organelle membranes before reacting with antigenic sites. These advantages apply equally to other antibodies, in particular nicotine and cotinine which are being developed in a parallel research program of this laboratory (please see Appendix).

Much of the chemistry required for our proposed studies is applicable for conjugation of nicotine or cotinine themselves (in addition to their antibodies) to microperoxidase (either 11-MP or 8-MP) for purposes of examining their fates by EM techniques. While a strong a priori argument can be made that nicotine or cotinine labelled intrinsically with tritium is more desirable than nicotine labelled extrinsically (as with 8-MP), the fact of the matter is that the former must be used in autoradiography whereas the latter can be used cytochemically. Cytochemical reactions almost invariably result in finer depositions at the level of electron microscopy. In addition, MP (the extrinsic label) is an enzyme and therefore provides through its activity a great amplification factor per molecule of nicotine and should greatly improve the opportunities for localizing small quantities of the drug in tissues prepared for EM.

2. Development of rapid, simple and sensitive assays of angiotensin converting enzyme (kininase II)

As stated above, it is now established that one of the pulmonary enzymes that degrades bradykinin (kininase II) is the same enzyme that converts angiotensin I to its active lower homolog, angiotensin II (angiotensinase converting enzyme). However, for the purposes of convenient discussion, the enzyme will be called converting enzyme. The point that we wish to emphasize is that the development of a rapid, simple and sensitive assay for studying the conversion of angiotensin I will yield data of equal importance to the inactivation of bradykinin.

Converting enzyme is a dipeptide carboxypeptidase of relatively little specificity. In addition to its ability to metabolize angiotensin I and bradykinin, it will also release the C-terminal dipeptides of hippuryl (Hip)-His-Leu, Hip-Gly-Gly, Z-Phe-His-Leu and the B-chain of insulin (for review, see Bakhle, 1974).

In previous studies on the purification of lung converting enzyme, Cushman and Cheung (1969) developed a synthetic substrate, Hip-His-Leu, that is resistant to non-specific "angiotensinase" enzymes while being highly reactive with converting enzyme. The substrate has the further advantage that the hippuric acid moiety is soluble in ethyl acetate while the substrate itself and the histidyl-leucine moiety are not. We have been especially attracted to the use of Hip-His-Leu because of the simplicity of solvent partitioning as a separation technique. However, the assay devised by Cushman and Cheung is not very sensitive: In their protocol, hippuric acid, released as a consequence of reaction of Hip-His-Leu with converting enzyme, is quantified by spectrophotometry (absorption at 218 nm).

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We propose to increase the sensitivity of the assay by labelling the hippuric acid moiety. Specifically, ^{14}C-glycine will be coupled to benzyl chloride to yield hippuric acid. The ^{14}C-hippuric acid will, in turn, be coupled to histidyl-leucine by carbodiimide condensation. We have synthesized the starting material, t-Boc-His-Leu-benzyl ester. After deprotection of the t-Boc- and after carbodiimide condensation, the benzyl ester will be removed by anhydrous HF or by saponification.

We envisage an assay protocol only slightly modified from that of Cushman and Cheung. After reaction of substrate with dispersed cells, the incubation medium will be acidified and extracted with ethyl acetate. The ethyl acetate layer (containing ^{14}C-hippuric acid) can be incorporated directly in scintillation fluid and counted.

The proposed assay should be sufficiently simple so as to allow essentially continuous measurements of the activity of converting enzyme in situ. For example, isolated lungs, prepared for single circulation experiments, can be cannulated via the left ventricle such that the pulmonary venous effluent is directed to a fraction collector. The test tubes of the fraction collector can be pre-loaded with an acid-ethyl acetate mixture to facilitate immediate separation of the hippuric acid moiety from undegraded Hip-His-Leu and from the second reaction product, His-Leu.

Thus it should be possible to subject the lungs to a variety of stimuli (e.g. under inflation, hyperinflation, hypoxia, hyperoxia, etc.) while measuring changes in the "flux rate" of the conversion of angiotensin I (or the degradation of bradykinin) at minute intervals. We use the term, "flux rate," as a general one to signify net changes in metabolism of the Hip-His-Leu substrate by whole lungs. Clearly, changes in the activity of converting enzyme may play only a minor role in these net changes; vasomotion, redistribution of blood flow, blood pressure, flow rates, etc., being much more important. However, for the concept of "endocrine" functions, the net changes are of paramount importance, as these are the changes which should determine how much angiotensin I, angiotensin II and bradykinin will enter the systemic arterial circulation and thus be supplied to target organs.

Significance

The ability of the lungs to process selectively certain circulating vasoactive substances has only recently come to light. In addition to the importance of these non-ventilatory functions to the lungs themselves, the conversion of angiotensin I to angiotensin II illustrates the ability of the lungs to produce substances which may influence organs at a distance. In this respect the lung is acting as an endocrine organ.

As mentioned previously it was evident from the beginning of our studies that we had discovered an entirely new mechanism of hormone metabolism. Nevertheless, there is little information on the effects of physiologic stimuli or environmental inhalants on these newly recognized functions or of the effects of failure by the lungs to carry out these functions.

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We propose to increase the sensitivity of the assay by [illegible] the hippuric acid moiety. Specifically, [illegible] ch Consequently, we have outlined a program for the definitive subcellular localization of angiotensin converting enzyme. In addition we plan to develop rapid flux assay methods for the detection of specific metabolites. While the conceptual and technical aspects of the program are ambitious, they present a rare opportunity. Success in the technical development of these studies will form the basis for determining the effects of drugs such as nicotine and of inhalants on a specific endocrine function of the lungs.

[illegible] incubation medium will be acidified and [illegible] ethyl acetate layer [illegible] directly in scintillation [illegible]

[illegible] essentially [illegible] measurements of the activity of converting enz[illegible]

[illegible]

[illegible] enzyme may play only a minor role in these net changes: vasomotion, [illegible]

[illegible] the systemic arterial circulation [illegible]

[illegible] conversion of angiotensin I to angiotensin II [illegible]

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9-52

APPENDIX: CONJUGATION OF NICOTINE, COTININE AND THEIR ANTIBODIES TO MARKERS FOR ELECTRON MICROSCOPY

Reference: See correspondence from Dr. R.C. Hockett to Dr. A. Castro, 2/15/74 (last paragraph) and from Dr. Castro to Dr. Hockett, 2/28/74.

Whereas a major aim of the research proposed in this application is to develop specific conjugation techniques for antibody to lung angiotensin converting enzyme, we wish to emphasize that the techniques to be developed should be immediately applicable for use with other antibodies and other antigens (e.g. nicotine, cotinine and their antibodies). In addition, we would like to point out that our laboratory has or will have on hand all of the necessary starting materials. Through a parallel research program (Nicotine in Blood: Detection by Radioimmunoassay), our staff have prepared 6-amino nicotine. This derivative was used for conjugation to bovine serum albumin (BSA) for immunization purposes and has also been used to prepare cotinine in a form suitable for conjugation to BSA. If we meet our immunization schedules, antibody to nicotine should be available by January 1, 1975, and antibody to cotinine should be available by May 1, 1975. These estimated dates of availability fit well with our schedule for developing new, specific techniques for conjugating antibody to the heme-octapeptide form of microperoxidase (8-MP).

We have a substantial quantity of 6-amino nicotine on hand. This derivative lends itself immediately to conjugation to microperoxidase using the N-hydroxysuccinimide polymer reacted with p-formylbenzoic acid (see text of this application). A nicotine-8-MP conjugate should be of considerable usefulness in studies of nicotine receptors at the level of electron microscopy and should supplement parallel studies using ^{3}H-nicotine in autoradiography.

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Space and facilities available (continued)

Equipment available by appointment (located in main Papanicolaou Building)

Philips EM 200 electron microscope
Balzer's freeze-etch apparatus
AMR SEM 900 scannning electron microscope

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Education:

Bristol University, Bristol, England 1960 - 1963

B.Sc. (Zoology - Special Honors, First Class)
B.Sc. (Chemistry - Subsidiary, First Class)
B.Sc. (Microbiology - Subsidiary, First Class)

Cambridge University, Cambridge, England 1968

Ph.D. (Cell Biology)

Research and/or Professional Experience:

1963 - 1967	Research Student, Department of Zoology, University of Cambridge, England (On leave of absence, 1964-1966)
1964 - 1966	Predoctoral Fellow, Department of Biology, University of Virginia, Charlottesville, Virginia
1966 - 1967	Predoctoral Fellow, Department of Medicine, University of Miami, Miami, Florida
1967 - 1971	Visiting Investigator, Laboratories for Cardiovascular Research, Howard Hughes Medical Institute, Miami, Florida
1970 - 1971	Director, Laboratory for Ultrastructure Studies, Howard Hughes Medical Institute, Miami, Florida
1967 - 1972	Instructor in Medicine, Department of Medicine, University of Miami School of Medicine, Miami, Florida
1968 - present	Adjunct Assistant Professor of Biology, Department of Biology, University of Miami, Miami, Florida
1971 - present	Assistant Professor of Medicine, Department of Medicine, University of Miami School of Medicine, Miami, Florida
1972 - present	Senior Scientist, Papanicolaou Cancer Research Institute, Miami, Florida

Academic and Professional Honors:

B.Sc. with First Class honors in Zoology, Chemistry and Microbiology
Bristol University Open Exhibition, 1960
U.K. State Scholarship, 1960
County Major Scholarship, 1960
D.S.I.R. Research Fellowship, 1963
Ethel Sargant Research Fellowship (awarded twice), 1964 and 1965
Science Research Council Research Fellowship, 1966
Established Investigator, American Heart Association, 1972 - present
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Interne		Montreal General Hospital McGill University	6/61 - 6/62
Assistant Resident, Medicine	Professor D. G. Cameron	As above	7/62 - 7/63
Research Associate	Dr. Seymour Kety	National Institute of Mental Health National Institutes of Health	7/63 - 9/65
Guest Investigator, Assistant Professor of Biochemistry (from April, 1968)	Professor R. B. Merrifield Dr. John M. Stewart	Rockefeller University New York, New York	9/67 - 9/68
Associate Professor of Medicine		University of Miami Miami, Florida	9/68 - present
Senior Scientist		Papanicolaou Cancer Research Institute Miami, Florida	9/72 present

Academic and Professional Honors:

Regional Scholarship - Dartmouth College, 1953-1957
Regional Scholarship - Cornell University Medical College, 1957-1961
Summer Research Traineeships, U.S.P.H.S., 1958-1959 - Cornell University Medical College
Smith, Kline and French Travelling Fellowship, 1960
William Mecklenberg Polk Research Prize, 1961 - Cornell University Medical College
Training award for studies in biological psychiatry, 1960-1961, Cornell University Medical College
Travel award, Rockefeller Foundation, 1962
Research Prize, Montreal Clinical Society, 1963
Postdoctoral Fellow, U.S.P.H.S., 1965-1967
Honorary Medical Officer to the Regius Professor of Medicine, Oxford, 1965-1967

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Career Development Award, U.S.P.H.S., March, 1968-September, 1968
Investigator, Howard Hughes Medical Institute, 1968-1971
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Visiting Professor, Clinical Research Institute of Montreal, January - June, 1974

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1962 - 65 The role of bradykinin in the pathogenesis of acute pancreatitis.
Supervisor: Dr. Alan G. Thompson, Montreal General Hospital, Montreal, Quebec.
Position: Research Associate.

1963 - 65 Studies of serum factors in schizophrenia.
Supervisor: Doctors Seymour Kety and Jack Durell, Clinical Center, National Institutes of Health, Bethesda, Maryland.
Position: Research Associate.

1965 - 67 Studies of renin in plasma and in solid tissues other than the kidney.
Supervisor: Professor Sir George W. Pickering, FRS, University of Oxford, Oxford, England.
Position: Honorary Medical Officer to the Regius Professor of Medicine.

1967 - 68 Metabolism of vasoactive polypeptides.
Supervisor: Dr. John M. Stewart, Rockefeller University, New York.
Position: Guest Investigator, September, 1967 - April, 1968;
Assistant Professor, April, 1968 - September, 1968.

1968 - 1) Metabolism of vasoactive polypeptides;
2) Non-ventilatory, endocrine functions of the lungs;
3) Renal hypertension. Howard Hughes Medical Institute, Investigator (1968-1971); Associate Professor of Medicine, University of Miami School of Medicine (1968 -) and Senior Scientist, Papanicolaou Cancer Research Institute

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11) Ryan, J.W., Steinberg, H.R., Green, R., Brown, J.D. and Durell, J.: Controlled study of the effects of plasma of schizophrenic and non-schizophrenic psychiatric patients on chicken erythrocytes. J. Psychiat. Res., 6:33, 1968.

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14) Ryan, J.W. and McKenzie, J.K.: Properties of renin substrate in plasma with a note on its assay. Biochem. J., 108:687, 1968.

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16) Ryan, J.W., Roblero, J. and Stewart, J.M.: Inactivation of bradykinin in the pulmonary circulation. Biochem. J., 110:795, 1968.

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30) Ryan, J.W. and Smith, U.: Metabolism of adenosine-5'-monophosphate during circulation through the lungs. Trans. Ass. Am. Physcns., 84:297, 1971.

31) Smith, U., Ryan, J.W., Michie, D.D. and Smith, D.S.: Endothelial projections: As revealed by scanning electron microscopy. Science, 173:925, 1971.

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CURRICULUM VITAE

Name: Andrew Tak-Chau Chiu

Birth date and place: REDACTED

Education:

Olivet College, Olivet, Michigan, 1966-1970, B.A. in Chemistry
University of Virginia, Charlottesville, 1970-1974, Ph.D. in Pharmacology

Research and/or Professional Experience:

1968-1970	Honors Program in chemistry, Olivet College, Olivet, Mich.
1968-1970	Chemistry Laboratory Instructor, Olivet College, Olivet, Mich.
1970-1974	Graduate student research in autonomic and endocrine pharmacology, University of Virginia, Charlottesville, Va.
1972-1973	Lecturer in pharmacology, Master and Practitioner Programs for Nursing Students, University of Virginia, Charlottesville, Va.
1973-present	Postdoctoral Fellow, Papanicolaou Cancer Research Institute, Miami, Fla.

Honors:

1969
1969
1970

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Publications:

1) Chiu, A.T.: Synthesis and study of decomposition rates of certain acyl-peroxides. Honors Thesis, Olivet College, Michigan, 1970.

2) Byrne, D.R., Burton, S.R., Chiu, A.T., Kabbe, F., Gruen, F.M. and Schuetz, R.D.: Synthesis and thermal decomposition of series of acyl-peroxides. J. Chem. Eng. Data, 17:507, 1972.

3) Peach, M.J. and Chiu, A.T.: The influence of acidosis on the chronotropic effect of tyramine in guinea-pig atria: relation of tyramine uptake and metabolism. J. Pharmac. Extp. Ther., 183:73, 1972.

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

June 25, 1974

Grant application No. 864B

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Feldman and Gardner

Subject: Theodore Alan Slotkin, Ph.D.
Continuation application No. 864B
"Catecholamine Stores in Normal and Hypertensive Rats"

History

CTR support of this investigator began in 1973, but has been awarded one year at a time. Hence the enclosed application requested "Continuation", meaning that it competes on the same basis as a new proposal.

Request

Application No. 864B requests $24,512 plus two additional years at approximately $17,000 each.

Documents Submitted

1. Enclosed is application dated 5/24/74 (16 pages).

2. Also enclosed is Progress Report #3, January 1 - June 30, 1974.

3. Reprints of publications identified as #1, 2, 5, 8, and 9 on page 3 of the Progress Report were provided; they will be forwarded to you promptly on request.

Comment

FWN visited this investigator on April 16, 1974; enclosed is copy of his report dated April 26, 1974.

We are seeking evaluation by an outside consultant.

F.W.N.

FWN:wg
Encls.

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CARDIOVASCULAR

~~RENEWAL~~ Continuation

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 5/24/74

1. Principal Investigator (give title and degrees):

Theodore A. Slotkin, Ph.D.
Assistant Professor of Pharmacology

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JUN 14 1974

2. Institution & address:

Duke University Medical Center
Durham, North Carolina 27710

3. Department(s) where research will be done or collaboration provided:

Department of Physiology and Pharmacology

4. Short title of study:

Catecholamine Stores in Normal and Hypertensive Rats

5. Proposed starting date: Jan. 1, 1975

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

In the previous 18 months of this project we have demonstrated the feasibility of studying sympatho-adrenal amine stores in the hypertensive rat (SHR) and during development in normal rats and have identified specific changes in synthesis, uptake, storage and secretion of catecholamines. During the time period outlined, we hope to:

1. Examine the effects of antihypertensive drugs in the SHR, including reserpine, aldomet, DBH inhibitors, α-methyl-p-tyrosine and 6-hydroxydopamine.
2. Examine the effects of acute and chronic nicotine administration in the SHR; nicotine is reputed to produce prolonged alterations in catecholamine turnover.
3. Detail the development of the adrenal medulla in the SHR to determine whether blood pressure changes precede or follow the alterations we have identified in catecholamine disposition.

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8. Brief statement of working hypothesis:

Studies from our and other laboratories have shown that catecholamine disposition is altered in the SHR, but do not indicate conclusively whether this is a causal factor in hypertension. By determining whether the sympatho-adrenal defects appear before or after the onset of hypertension (maturation studies) we may obtain an answer to this question.

We have demonstrated that the effects of drugs which act on storage vesicles are altered in the SHR. These factors may contribute to similar difficulties observed in the therapy of human essential hypertension with these agents. The proposed study will identify how the actions differ and may suggest alternative therapies.

The sympatho-adrenal system of the SHR is set on a "hair trigger" and hyperresponds to small stress inputs. This suggests that chronic administration of nicotine may produce profound alterations in catecholamine stores of the SHR, which in turn could markedly affect cardiovascular function.

9. Details of experimental design and procedures (append extra pages as necessary)

see appended pages 11 - 16

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory consists of 850 square feet fitted with standard laboratory type benches. Major items of equipment include: Sorvall RC-2B centrifuge, Beckman L5-50 ultracentrifuge and rotors, Farrand ratio fluorometer, catecholamine autoanalyzer, Wang 600-6-TP programmable calculator, incubation baths, pH meter, balances and other general items of laboratory hardware. Research facilities include animal rooms, cold rooms and a spectrophotometer.

11. Additional facilities required:

Funds are requested to purchase a Beckman liquid scintillation counter. We have previously made use of a counter in another department, but as all our assays are radiochemical, it has become increasingly difficult to borrow enough counter time to accomodate our needs. This situation has hampered our ability to obtain results in time to plan future experiments on schedule.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

See pages 6, 7, 8 and 9.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See page 10.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)

	% time	Amount
Theodore Slotkin - Principal Investigator		REDACTED
Technical		REDACTED
Frederic J. Seidler (B.S.)		
Fringe Benefits @ 12.45%		
Sub-Total for A		REDACTED

B. Consumable supplies (by major categories)

Animals, including housing & shipping costs	2500
Isotopes	1500
Chemicals and Hardware	1000
Sub-Total for B	5000

C. Other expenses (itemize)

Equipment maintenance and service	500
Travel	500
Sub-Total for C	1000
Running Total of A + B + C	$14097

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D. Permanent equipment (itemize)

Beckman liquid scintillation counter (see item 11 on page 3)	8300
Sub-Total for D	8300
E	2115
Total request	$24,512

E. Indirect costs (16% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	8583	5000	1000	0	2188	16,771
Year 3	9098	5000	1000	0	2265	17,363

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Amine stores of developing normal & hypertensive rats*	American Heart Association #73-611	$50,000	10/1/73 - 9/30/76
2. Effects of morphine on the adrenal medulla†	NIMH, DA-00465	85,000	6/1/73 - 5/31/76
3. Faculty development award§	Pharmaceutical Manufacturers Association Foundation	40,000	7/1/73 - 6/30/75

* This project is directed primarily toward maturation studies and funds are mostly for salary support and pregnant rats. The current request is mandatory for continuation of studies on effects of reserpine, nicotine, etc. in adult SHR and to offset the rising costs of working with SHR. For example, one SHR rat costs $7.50 and one pregnant SHR $25.
† Unrelated to proposed project.
§ Salary support for principal investigator.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Theodore A. Slotkin

Signature Theodore Slotkin Date

Telephone 919 684-5224
Area Code Number Extension

Checks payable to

Duke University

Mailing address for checks

Mr. C. B. Huestis, V. P., Business & Finance
203 Allen Building, Duke Universtiy
Durham, North Carolina 27706

Responsible officer of institution

Typed Name W. G. Anlyan, M.D.

Title Vice President for Health Affairs

Signature W G Anlyan Date 6/11/74

Telephone 919 684-5175
Area Code Number Extension

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CURRICULUM VITAE

NAME:
Theodore Alan Slotkin

BORN: REDACTED

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REDACTED

REDACTED

EDUCATION AND DEGREES:
B.S. - Brooklyn College, CUNY, 1967
Ph.D. - Department of Pharmacology and Toxicology - University of Rochester, 1970

POSITIONS HELD:

June 1971 - present	Assistant Professor, Department of Physiology and Pharmacology, Duke University Medical Center
June 1970 - May 1971	Research Associate, Department of Biochemistry, Duke University Medical Center
February 1970 - May 1970	Postdoctoral Fellow, Department of Pharmacology and Toxicology, University of Rochester

SOCIETIES:

REDACTED

RESEARCH ACTIVITIES:
Neurochemistry
Neuropharmacology
Hypertension

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Slotkin, T.A., DiStefano, V. and Au, W.Y.W. Blood levels and urinary excretion of harmine and its metabolites in man and rats. J. Pharmacol. Exp. Ther. 173:26-30 (1970).

Slotkin, T.A. and DiStefano, V. A model of harmine metabolism in the rat. J. Pharmacol. Exp. Ther. 174:456-462 (1970).

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Slotkin, T.A. and Green, H.O. Drug-resistant effect of adenine nucleotides and magnesium on catecholamine efflux from isolated adrenal medullary storage vesicles. Biochem. Pharmacol. in press.

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Slotkin, T.A. and Green, H.O. Adrenal Medullary Storage Vesicles of the Spontaneously Hypertensive Rat. Biochem. Pharmacol. submitted.

Slotkin, T.A. Structure-Activity Relationships for the Reserpine-Like Actions of Derivatives of β-carboline in vitro. Life Sci., submitted.

Anderson, T.R. and Slotkin, T.A. Acute and Chronic Effects of Morphine on Rat Adrenal Medulla. Biochem. Pharmacol. submitted.

Mills, E. and Slotkin, T.A. Oxygen-Dependent Changes in Catecholamine Content of the Carotid Body. Science, submitted.

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ABSTRACTS

Slotkin, T.A. and DiStefano, V. Urinary metabolites of harmine in the rat and their inhibition of monoamine oxidase. Fed. Proc. 28:797 (1969).

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13. Five most recent and pertinent articles:

1. T.A. Slotkin and H.O. Green. Adrenal Medullary Storage Vesicles of the Spontaneously Hypertensive Rat. Biochem. Pharmacol., submitted.

2. T.A. Slotkin. Maturation of the adrenal medulla. III. Practical and Theoretical Considerations of Age-dependent Alterations in Kinetics of Incorporation of Catechol- and Non-catecholamines. Biochem. Pharmacol., in press.

3. H.O. Green and T.A. Slotkin. Reserpine-like effects of harmine on isolated adrenal medullary storage vesicles. Mol. Pharmacol. 9:748 (1973).

4. T.A. Slotkin. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. Biochem. Pharmacol. 22:2033 (1973).

5. T.A. Slotkin. Maturation of the adrenal medulla. I. Uptake and storage of amines in isolated storage vesicles of the rat. Biochem. Pharmacol. 22:2023 (1973).

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9. Details of experimental design

A. Introduction

The adrenal medulla is often utilized as a model of the sympathetic neuron; both tissues arise embryonically from the neural crest and both have the ability to synthesize, store and secrete catecholamines. Each contains storage vesicles which can accumulate amines by a mechanism which is stimulated by ATP-Mg^{2+} and blocked by reserpine. The vesicles contain dopamine beta-hydroxylase (DBH), chromogranins and adenine nucleotides as well as catecholamines; it is accepted generally that the catecholamines and adenine nucleotides (primarily ATP) form a storage complex in a molar ratio of 4 to 1.

During prenatal and postnatal development, there is a marked increase in catecholamine levels in adrenergic neurons and in the adrenal medulla (1-5), as well as changes in catecholamine synthesizing enzymes (1,6). Although the necessary enzymes are present early in gestation, catecholamines do not appear until late in gestation, at a time when storage vesicles first become detectable (7,8), suggesting that the storage vesicles play a determining role in the increase in adrenal amines. Consequently, the largest changes in catecholamine content occur in the postnatal period (0-6 weeks after birth). Spontaneously hypertensive Wistar rats (SHR) first show significantly elevated blood pressures towards the end of this period (5 weeks), along with disturbances in sympathetic catecholamine synthesis, storage and release (9). Similarly, in studies utilizing uninephrectomized rats treated with desoxycorticosterone acetate and NaCl, it has been shown that the resultant hypertension is accompanied by a defect in catecholamine storage such that cardiac storage vesicles become "leaky"(10). Westfall(11) has likewise demonstrated changes in catecholamine turnover in rats with elevated systolic blood pressures produced by chronic nicotine administration. Impairment of catecholamine storage has been implicated in essential hypertension in humans, as evidenced by increases in excretion of catecholamines and their metabolites in individuals with that disease (12).

In each case, hypertension was accompanied by a disturbance in sympathetic function possibly involving impaired storage. Therefore, it is important to examine systematically the properties of the storage vesicles in at least one of the model systems. The SHR is probably the most reliable of all methods of hypertension to use for a study of this type: hypertension develops rapidly and requires no pharmacological or surgical intervention, the animals are available as a genetically pure strain, and inbred normotensive Wistar rats provide a valid control (9). It is of additional interest that, although the mechanism of hypertension may be different from SHR, essential hypertension in humans is probably genetic in origin (13).

While it is of obvious importance to determine the mechanisms involved in the age-dependent increases in sympatho-adrenal amines and the development of altered storage in SHR, few studies have been published on the properties of the storage vesicles during these changes. Brundin (14) measured the rate of efflux of endogenous

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amines from isolated neonatal rabbit adrenal vesicles, but did not do simultaneous studies utilizing vesicles from adults; furthermore, he reported only two time points (30 and 60 minutes), while several studies have shown that amine efflux is biphasic and therefore requires kinetic analysis of many time points to obtain a valid picture of amine storage (15-19). Mirkin (5) has studied the storage properties of vesicles from fetal and developing rat heart; these studies utilized measurements of in vivo uptake or uptake in the isolated, perfused heart. Thus, the observed development of uptake or storage might have reflected proliferation of adrenergic neurons in the heart, or changes in the uptake of amines across the axonal membrane, rather than alterations in the storage vesicles per se. The studies by Mirkin (5) also indicate the difficulties of working with adrenergic neurons compared to the adrenal medulla; a single density gradient experiment required the pooling of 50-60 fetal or neonatal rat hearts. Obviously, experiments of this type are not feasible if large numbers of observations need to be made. For this reason, the adrenal medulla provides a more useful model than sympathetic nerves with which to study amine storage. Purified adrenal vesicles can be obtained in high yield by a relatively rapid discontinuous density gradient technique (20), while much lower yields of comparably purified sympathetic nerve vesicles are obtained after more lengthy procedures. Furthermore, the high concentration of storage vesicles in the adrenal permits the evaluation of properties which would be far more difficult to determine in nerve vesicles.

Similarly, because of the difficulty in evaluating the properties of nerve storage vesicles, most of the studies on catecholamine turnover in hypertension have involved whole animal or whole organ studies (9-13). There is, however, one report of altered cardiac vesicular amine storage in which elevated ratios of extragranular to intragranular amines were observed in hypertensive rats (10). These data further reinforce the need for studies which can identify specific alterations in vesicle properties.

For the past four years, the research of this investigator has been concerned with the development of sensitive and appropriate methods for the evaluation of the properties of the catecholamine storage vesicles of the adrenal medulla (16-19). By using these techniques, the following parameters can be measured:

a. Uptake and storage capabilities of the vesicles. The simultaneous measurement of the accumulation of radioactively labelled amines by the vesicles along with the efflux of endogenous and labaled amines permits evaluation of these two parameters. The rate of efflux is determined by the stability of storage, while the accumulation is a measure of storage stability and affinity for uptake. Additionally, the relative importance of ATP-Mg^{2+} stimulated uptake can be evaluated by measuring the accumulation of metaraminol, an amine which is incorporated by a primarily ATP-Mg^{2+}-independent mechanism (16, 17).

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b. Concentrations of intravesicular components. Vesicles are purified by discontinuous sucrose density gradient centrifugation. The subcellular distributions of catecholamines, ATP and DBH can thus be readily determined, along with the fragility of the vesicles. The buoyant density of the vesicles can also be studied by continuous density gradient centrifugation, which provides a sensitive measure for evaluating small differences in the densities of different populations of vesicles.

c. Secretion and recovery of amines and vesicles. This can be evaluated using neurogenic secretion evoked by insulin-induced hypoglycemia or non-neurogenic amine loss produced by reserpine.

Using these techniques, work supported by the Council for Tobacco Research has been conducted by the investigator over the last 18 months on the development of the adrenal medulla in NWR and on the alterations in catecholamine disposition in adult SHR. The results have been summarized in detail in the progress reports and in publications from this project (see list), but a brief listing appears below:

1. The maturation process is accompanied by profound changes in the uptake, storage, synthesis and release of adrenomedullary catecholamines; various markers have been found to study the process in the projected studies for SHR.

2. Adult SHR show an adrenal catecholamine biochemical pattern typical of chronic understimulation; however the sympatho-adrenal axis is hyperresponsive to ordinarily mild stress stimuli.

3. Alterations in the storage vesicle membrane of the SHR result in abnormal responses to antihypertensive drugs and may alter the effects of agents which increase catecholamine turnover, such as nicotine.

In the proposed extension of this study, we intend to determine in detail:

1. The effects of chronic and acute nicotine administration in NWR and SHR.

2. The effects of chronic and acute administration of antihypertensive agents.

3. The time course of development of hypertension and changes in catecholamine disposition during maturation of the SHR.

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These studies will identify whether sympatho-adrenal changes cause or are caused by the changes in cardiovascular dynamics and will demonstrate whether drugs which affect catecholamine disposition can improve or worsen the defects in the SHR.

B. Methods

In studies with antihypertensive drugs, adult SHR and NWR will be given single or repeated injections of reserpine, α-methyldopa (Aldomet), DBH inhibitors (disulfiram and fusaric acid), α-methyl-p-tyrosine and 6-hydroxydopamine (acute only). Appropriate doses for acute and chronic administration of all of these agents have already been worked out and published in our and other laboratories. In some experiments, chlorisondamine, a long-acting ganglionic blocking agent, will be administered to interrupt sympathetic reflex activity and enable studies of direct actions of each drug. SHR and NWR will be sacrificed at intervals of 4 and 24 hr after initial administration, at intervals of several days during chronic administration, and several weeks after acute administration of 6-hydroxydopamine.

Studies with nicotine will involve acute and chronic (twice daily) doses of 2.5 mg/kg s.c. and 25 mg/kg s.c. for periods up to 2 months; animals will be sacrificed at weekly intervals.

Studies with SHR neonates will involve sacrifice at 10-day intervals from birth to 50 days of age; blood pressure will be monitored by tail plethysmography (20). In some studies, SHR and NWR neonates will be chemically sympathectomized with 6-hydroxydopamine and subsequent changes in blood pressure and catecholamine disposition will be monitored.

After sacrifice, adrenals will be removed, cleaned of connective fat and tissue, and homogenized in isotonic sucrose. Subcellular fractions will be obtained by differential and density gradient centrifugation. The detailed methodologies will not be described here because they appeared in our previously funded CTR applications; for this information, please refer to the appended reprints and manuscripts. We will make the following determinations:

1. Activities of catecholamine biosynthetic and degradative enzymes (tyrosine hydroxylase, dopa decarboxylase, dopamine β-hydroxylase and monoamine oxidase).

2. Catecholamine levels in the whole tissue.

3. Subcellular distributions of catecholamines, ATP and dopamine β-hydroxylase.

4. Buoyant density and fragility of storage vesicles.

5. Ability of storage vesicles to incorporate ^{14}C-epinephrine or ^{3}H-metaraminol in terms of:

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a. Uptake capacity per gland.
b. Uptake capacity per vesicle.
c. Affinity and maximal transport rate for each amine.

6. Efflux of endogenous and newly-incorporated amines.

7. Functional rate of β-hydroxylation in intact vesicles.

These data will enable us to identify specific changes in catecholamine disposition in terms of hypertension or drug-induced changes in synthesis, uptake, storage or secretion in a fashion analogous to our previous work in developing NWR and adult SHR, and to establish whether the actions of nicotine and antihypertensives differ in the SHR compared to NWR.

C. Rationale and significance.

The development of hypertension in spontaneously hypertensive rats, in rats with surgically or pharmacologically induced hypertension, in human essential hypertension, and in rats chronically treated with nicotine, is associated with alterations in catecholamine disposition. The sympathetic nervous system and its endocrine counterpart, the adrenal medulla, exert important regulatory functions on the entire cardiovascular system. During the first six weeks after birth, the adrenergic neurons and adrenal medulla of the rat undergo marked changes in catecholamine synthesis, uptake, storage and release. At the same time, hypertension begins to develop in spontaneously hypertensive Wistar rats, which is associated with defects in the physiological disposition of sympathetic amines. The proposed study is designed to identify at what time after birth these changes occur. The earlier studies from our lab have shown that some of the sites of action of antihypertensive drugs and nicotine are altered in the SHR. The proposed studies will identify the differences in the actions of these agents in SHR and NWR, which could be of considerable significance in evaluating the potential exacerbation of sympatho-adrenal alterations by nicotine as well as the development of refractoriness to antihypertensive drugs.

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Progress Report no. 3
Jan. 1, 1974 - June 30, 1974

CTR Grant #864
Theodore Slotkin
Department of Physiology and Pharmacology
Duke University Medical Center
Durham, North Carolina 27710

MATURATION OF THE ADRENAL MEDULLA: CATECHOLAMINE STORES IN NORMAL AND HYPERTENSIVE RATS

The first progress report from this project detailed the biochemical maturation of the adrenal medulla in normotensive Wistar rats (NWR) and in the second report these changes were identified on the molecular level and reduced to a set of mathematical parameters describing the development process. Additionally, studies were commenced utilizing similar techniques to evaluate adrenal catecholamine synthesis, uptake, storage and release in adult spontaneously hypertensive rats (SHR). To determine whether increased sympatho-adrenal activity plays a role in the development of hypertension, the studies concentrated the properties of the adrenomedullary storage vesicles.

The uptake of ^{14}C-epinephrine per 100 µg of endogenous catecholamines in isolated SHR vesicles was higher than in NWR while the uptake of ^{3}H-metaraminol was the same as in NWR; thus, SHR vesicles exhibited a higher preference for epinephrine vs. metaraminol compared to NWR. The difference in uptake was due to a lower Km for epinephrine in SHR. The storage of amines was the same in SHR and NWR, as demonstrated by measurements of catecholamine to ATP ratios in purified vesicles, and effluxes from the vesicles of endogenous and newly-incorporated amines. The ratio of catecholamines to dopamine β-hydroxylase (DBH, a marker for storage vesicles) was higher in SHR for three reasons: (1) there were fewer vesicles per gland; (2) there was less DBH per vesicle, indicated by an increased precursor/product ratio ; and (3) there was a higher catecholamine content per vesicle, as shown by an increase in the ratio of heavy to light vesicles on discontinuous sucrose density gradients. SHR adrenals were depleted of catecholamines after insulin administration to a greater extent than were NWR adrenals, and both SHR and NWR exhibited induction of tyrosine hydroxylase and dopamine β-hydroxylase after insulin. None of these findings is consistent with the view that sympatho-adrenal hyperactivity occurs in the SHR; the data suggest that hypoactivity occurs, perhaps secondarily to the hypertension. However, the sympatho-adrenal axis of the SHR appears to be set on a "hair trigger" such that stimuli which usually cause a small or moderate discharge result in a much larger discharge in the SHR. This may be of some significance in evaluating the effects of chronic, low level stress in the SHR, such as that produced by low doses of nicotine (see discussion of future experiments).

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Our latest studies have tested the effects of the antihypertensive drug, reserpine, in SHR and NWR. Reserpine elicits a triphasic response -- initially there is a reflex stimulation of the sympatho-adrenal axis similar to the effect of insulin. This is followed by catecholamine depletion from reserpine blockade of the vesicular uptake mechanism; thirdly, there is a late increase in stimulation resulting from the hypotensive effect of the drug. We have found that the SHR is more susceptible to the initial and late stimulatory phases of reserpine action, as would be expected from the hypersecretion obtained from insulin. The differences between NWR and SHR in these phases can be eliminated by administration of the ganglion blocker, chlorisondamine. When this is done, SHR appear to be less sensitive to the depletion effect of reserpine, probably as a result of the alteration in the Km for uptake of epinephrine. If human essential hypertension is in fact similar to the SHR, these data might explain the development of refractoriness to antihypertensive medications which act on the level of the storage vesicle (reserpine, aldomet).

One important question which was unresolved was whether the hypersecretion on stimulation of the SHR adrenal resulted from a central effect (increased frequency of action potentials coming down the splanchnic nerve) or whether the gland hyperresponds to the same number of stimuli. To test this hypothesis, direct secretion was evoked by nicotine administration. Under these conditions, an equivalent secretory response was obtained in SHR and NWR, indicating that the defect is central in origin.

Over the next 6 months we expect to extend these findings to cover other important antihypertensive medications (aldomet, DBH inhibitors, α-methyl-p-tyrosine, 6-hydroxydopamine) and to commence further studies on the acute and chronic effects of low and high doses of nicotine, which is reputed to cause persistent alterations in autonomic control of blood pressure; our preliminary work on acute nicotine administration suggests that the SHR is not hypersensitive to the drug, but other investigators have shown that alterations in catecholamine turnovers usually appear only after chronic administration. Obviously, chronic studies are required to see if differences appear in the SHR vs. the NWR.

In evaluating these changes, it is apparent that one must differentiate between those which are involved in the hypertensive process and those which are unrelated ancillary genetic alterations. For this reason, we originally intended immediately to examine the development of amine stores and hypertension in the maturing SHR, to see whether sympatho-adrenal changes precede or follow the development of high blood pressure. Because of delays in obtaining a reliable source of pregnant SHR (there is only one commercial supplier), these studies will be delayed until this winter; Carworth Farms has assured us the animals will be available at that time.

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Publications from this project:

1. Maturation of the adrenal medulla. I. Uptake and storage of amines in isolated vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. 22:2023-2032 (1973).

2. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. 22:2033-2044 (1973).

3. Binding of amines to purified bovine adrenal medullary storage vesicle membranes. T.A. Slotkin and N. Kirshner, Biochem. Pharmacol. 22:2492-2497 (1973).

4. Hypothetical model of catecholamine uptake into adrenal medullary vesicles. T.A. Slotkin, Life Sci. 13:675-683 (1973).

5. Reserpine-like effects of harmine on isolated adrenal medullary storage vesicle. H.O. Green and T.A. Slotkin, Mol. Pharmacol. 9:748-755 (1973).

6. Secretion and recovery of catecholamines by the adrenal medulla. N. Kirshner and T.A. Slotkin, Biochem. Pharmacol. in press.

7. Drug-resistant effect of adenine nucleotides and magnesium on catecholamine efflux from isolated adrenal medullary storage vesicles. T.A. Slotkin and H.O. Green, Biochem. Pharmacol., in press.

8. Maturation of the adrenal medulla. III. Practical and theoretical considerations of age-dependent alterations in kinetics of incorporation of catechol- and non-catecholamines. T.A. Slotkin, Biochem. Pharmacol., in press.

9. Adrenal medullary storage vesicles of the spontaneously hypertensive rat. T.A. Slotkin and H.O. Green, Biochem. Pharmacol. in press.

10. Structure-activity relationships for the reserpine-like actions of derivatives of beta-carboline. T.A. Slotkin, Life Sci., in press.

Manuscripts in preparation:

1. Effects of acute administration of reserpine and nicotine on catecholamine stores of the spontaneously hypertensive rat. T.A. Slotkin.

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2. Effects of tryptamine and phenethylamine derivatives on catecholamine uptake into isolated rat adrenal medullary vesicles. T.A. Slotkin, Frederic J. Seidler and Martha D. Abou-Donia.

Abstracts:

1. Uptake and storage of amines in isolated adrenal medullary vesicles vesicles of developing rats. T.A. Slotkin, Fed. Proc. 32:783Abs (1973).

2. Maturation of adrenal catecholamine storage vesicles of the rat. T.A. Slotkin, Pharmacologist 15:210 (1973).

3. Adrenal Medullary Vesicles of Hypertensive Rats. T.A. Slotkin and H.O. Green, Clin. Res. 22:13A (1974).

4. Effects of tryptamines on epinephrine uptake into adrenal medullary vesicles. T.A. Slotkin, F.J. Seidler and M.D. Abou-Donia, Pharmacologist, in press.

Theodore Slotkin

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#833A - WELTMAN

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 12, 1974

Grant Application No. 833A

CARDIOVASCULAR

To: The committee comprising Drs. Bing, Gardner and Sommers

Subject: A. Stanley Weltman, Ph.D., Brooklyn College of Pharmacy, Long Island University, Brooklyn, N.Y.
Continuation application No. 833A
"Effects of Nicotine and Cholesterol in Spontaneously Hypertensive and Normotensive Rats"

History

CTR began support of this investigator in 1972. Renewal for 1974 was requested but denied. Recently an inquiry from Dr. Weltman concerning renewed support, with some reorientation of the study, was handled as Case No. 271. The Executive Committee expressed reservations but voted to encourage a full proposal.

Request

Application No. 833A requests $37,117, plus one additional year.

Documents Submitted (attached)

1. Application dated 6/25/74.
2. Abstracts:
   a. "Nicotine Effects in Spontaneously Hypertensive Rats," Weltman, et al, Federation Proceedings 1973.
   b. "Chronic Nicotine Effects in Spontaneously Hypertensive Rats," Weltman, et al, Federation Proceedings, 1974.
   c. "Acute Effects of Nicotine in Spontaneously Hypertensive Rats," Weltman, et al, Academy of Pharmaceutical Sciences, 1974.

Comment

Drs. Sommers and Nordsiek visited Dr. Weltman some time ago. The general impression was of a well run laboratory doing careful and thorough, if somewhat unimaginative, work.

We are seeking an outside reviewer to evaluate this proposal.

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#133A

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8883

RECEIVED
JUL 1 1974

Application for Research Grant
(Use extra pages as needed)

Date: 6/25/74

1. Principal Investigator (give title and degrees):

A. Stanley Weltman, Ph.D., Associate Professor in Pharmacology and Research

2. Institution & address:
Laboratories for Therapeutic Research
Brooklyn College of Pharmacy
Long Island University
600 Lafayette Avenue, Brooklyn, N. Y. 11216

3. Department(s) where research will be done or collaboration provided:
a) Laboratories for Therapeutic Research
b) Institute of Pathology, Downstate Medical Center, S. U. N. Y.
Brooklyn, N. Y.

4. Short title of study:
Effects of Nicotine and Cholesterol in Spontaneously Hypertensive and Normotensive Rats

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Two Years

7. Brief description of specific research aims:

As subsequent discussion will illustrate, considerable controversies exist concerning possible deleterious effects of the habit of smoking (nicotine) and/or high cholesterol diets on hypertension, cardiovascular, arteriosclerotic and atherosclerotic pathologies. One can question the progressive effects of these agents not only in normal individuals but those subject to essential hypertension. The Japanese strain of spontaneously hypertensive rats (SHR) has been reported to represent an excellent animal model of essential hypertension. The present investigation therefore proposes to compare and relate interactions of intermediate and prolonged periods of either single and/or combined administration of nicotine alkaloid and high cholesterol diets with systolic blood pressure, plasma lipid profile and the possible etiological development of hypertension, arteriosclerosis and atherosclerosis. Animals to be utilized will be the spontaneously hypertensive rats as well as its comparable genetic normotensive strain. Among the many questions to be resolved are the possible synergistic and antagonistic actions etc., of either the single and/or combined treatments on the blood lipid profile, blood pressure and vascular pathologies, etc. in the SH and normotensive strains. Doses employed will be 2.28 mg/kg/day of nicotine alkaloid administered in drinking water (equivalent to 2 packs of cigarettes per day) and a Purina Lab Chow Meal mix containing an additional 5% cholesterol supplement.

In previous studies, our laboratory has demonstrated that 2.28 mg/kg/ of nicotine alkaloid administered to SHR for 6 and 29 weeks caused decreases in body weights, consistent trends of lower systolic blood pressure levels by the 4th week and significant decreases in plasma cholesterol levels of rats sacrificed after 6 and 29 weeks

(SEE ATTACHED PAGES 6-13)

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8. Brief statement of working hypothesis:

Our present proposal aims to determine if nicotine decreases cholesterol, affects lipid metabolism and diminishes hepatic cholesterol synthesis in the spontaneously hypertensive rats. Concomittant studies would explore the effect in genetically related normotensive male rats. In addition, the study proposes to clarify interactions and effects of nicotine and cholesterol intake in the progressive development of hypertension in initially hypertensive and normotensive animals.

Considerable controversy exists concerning the effects of smoking and/or nicotine on cholesterol levels as well as the relationships of high cholesterol intake with the development of hypertension, vascular pathology, atherosclerosis etc. Several investigators have reported higher cholesterol levels in smokers. Findings by Svacha et al (58) of effects of egg intake and tobacco smoking on serum cholesterol levels appeared to indicate that serum cholesterol responses to egg intake was amplified by smoking. While numerous reports have indicated associationships of cholesterol with hypertension, vascular pathology, atherosclerosis etc., Nagaoka et al (114) claimed that feeding of high cholesterol diets (2%) plus 0.15% propylthiouracil or a diet of 2% cholesterol to spontaneously hypertensive rats indcated that cholesterol was not involved in the progression of hypertension nor in the occurrence of thrombus formations.

(see attached page 14)

9. Details of experimental design and procedures (append extra pages as necessary)

Mature male spontaneously hypertensive rats (SHR-Series I) and genetically related normotensive Wistar rats (WKY/N-Series II) approximately 10 weeks of age will be selected for the 6 month and 1½ year studies. Within each series, the hypertensive and normotensive animals will be matched by body weights and divided into 4 groups: Group A-nicotine-treated (2.28 mg/kg/day); Group B-cholesterol (5%); Group C-nicotine and cholesterol regimens; Group D-untreated controls. The rats in each group will be housed 4 per cage in duprolene cages (22" x 15" x 7") provided with stainless steel lids with feed and water compartments and a removable stainless steel grid floor. Trays placed beneath the hanging cages and provided with Sani-chips will permit sanitary maintenance of the cages. During an initial two week period, animals will be permitted to eat and drink water ad libitum. Water consumption will be measured daily via the use of graduated water bottles. Measured quantities of food will be provided to all groups consisting of Purina Laboratory Chow Meal and the total food consumption and water consumption will be evaluated during the respective weekly periods. Analysis of food consumption and body weight gain relationship obtained via weekly body weight measurements will permit evaluation of food utilization ratios (body weight gain (gm)/food consumed (gm) in the respective treated and control spontaneously hypertensive groups.

After the first week of acclimatization, base line studies of systolic blood pressures will be obtained. The indirect measurement of systolic pressure will be obtained via the tail-cuff procedure and by means of a pulse transducer (146). Readings will be obtained with a Narco-Biosystems Physiograph (Desk Model-DMP-4B) from warmed, unanesthetized rats. Prior to placing the individual rats in a Narco-Biosystems Inc. plastic restraining cage warming board unit (37°C), all animals will be individually prewarmed in an incubator maintained at 37°C for 15 minutes. As employed at our laboratory blood pressure levels will be based on averages derived from 10 readings.

At the completion of the 2 week period and at approximately 12 weeks of age, nicotine and cholesterol treatment will be initiated for the respective groups. On the basis of instructions for chronic administration of nicotine used by the Dr. Wenzel laboratory (private communication), the daily dose of nicotine will be provided in a volume of water that the group of animals will consume in approximately 18 hours, taking into account the volume of water lost by dripping. Our laboratory provides the proper amount of drinking water with nicotine to the animals at 3:00 pm and at 9:00 am the following morning water bottles are checked, water consumption estimated and nicotine-containing bottles, replaced with measured quantities of fresh tap water for the remaining 6 hours. During the 18 hour period on the basis of water consumption and body weight measurements nicotine alkaloid drinking solutions will be replaced to provide a daily oral intake of 2.28 mg/kg body weight

(see attached pages 15-24)

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

a) The space and facilities available at the Laboratories for Therapeutic Research, Brooklyn College of Pharamcy are as follows:

The Laboratories were designed for the purpose of conducting animal investigations in physiology,pharamacology, endocrinology, biochemistry, and experimental therapeutics. It is a Laboratory which is equipped for work in all of these fields and possesses histological, microscopic, biochemical and animal-surgical equipment necessary for the conduct of detailed investigations.

The animal rooms are air-conditioned, the animals are housed in metal cages and an automatic cage-washing machine is available. The permanent equipment in addition to the cages includes: (1) Leitz Ortholux Binocular Microscope, (1) Sartorius Selecta Precision balance, (1) F.P.E. Precision balance, (2) ovens, (2) Incubators, (2) Refrigerators, (1) Freezer, (1) Turner Fluorometer, Model 110, (1)Coleman Spectrophotometer Model 6, (1) Spectronic 20 (Bausch & Lomb), (1) Servall Centrifuge, (1) Adams Dynac Centrifuge, (1) Torbal Torsion Balance, (1) Bausch & Lomb freezing and (1) Spencer rotary paraffin microtome, a Technicon for processing histological specimens, (1) Beckman pH Meter, (2) A.H. Thomas shakers, (1) Demineralizer Unit (Barnstead), (1) Corning AG-1 Glass Distilling Apparatus, (1) Hot plate, (1) Stir-Jack, (1) Elconap Constant Temperature Water Bath, (1) Friden Calculator, (1) Friden 130 Electronic Calculator, (1) Marchant Cogito 566 PR Calculator, (1) General Radio Oscillator, Type 1210 C and amplifier, (1) Audiogenic Stress Belling Chamber, (1) Stainless Steel Pipette Washer and a miscellany of glassware and accessory equipment. (1) Narco-Biosystems, Desk Model DMP-4B, Physiograph and accessory equipment for systolic blood pressure measurements. In addition to a fluorometer, spectrophotometer and a Micrometric Syringe Microburet (Model SB 22) equipment for the various biochemical

(see attached page 25)

11. Additional facilities required:

12. Biographical sketches of investigator(s) and other professional personnel (append):

A.S. Weltman (pages 26-30), N.Y. Mary (pages 31,32), V.M. Yermakov (pages 33-35), S. Schwan (pages 36,37)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

(see page 38)

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
A. Stanley Weltman, Ph.D.	25	N.C.
Nouri Y. Mary, Ph.D.	15	N.C.
*Valentin M. Yermakov, M.D.	10	N.C.
*Stefan Schwan, M.D.	15	N.C.
Technical		
Vijay Pandhi, M.S.	100	13,000
Leroy Johnson, B.S.	75	7,500
Ratilal Vaidya, B.S.	50	5,000
Caretaker	30	2,210
(Pathology Technicians Pending Supplemental needs)	---	------
Sub-Total for A		$ 27,710

* Pending agreement to pursue pathological study, as a supplemental grant after completion and evaluation of biochemical and blood pressure data (see section 9 page 16)

B. Consumable supplies (by major categories)	
Feed and Bedding	850
SHR and WKY/N rats (Normotensive)	1,040
Glassware, Chemicals, Lipoprotein assays etc. Physiograph and Densitometer Recording Papers, etc.	1,675
Sub-Total for B	$ 3,565

C. Other expenses (itemize)	
Sub-Total for C	
Running Total of A + B + C	
D. Permanent equipment (itemize)	
Animal Rack and Cages	1,000
Sub-Total for D	$ 1,000
E. Indirect costs (15% of A+B+C)	4,842
Total request	$37,117

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$27,710	$2,800			$4,577	$35,087
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Laboratories for Therapeutic Research Research Institute of The Brooklyn College of Pharmacy, is a non-profit basic research institution at Brooklyn College of Pharmacy. Costs of Plant Operation are jointly shared by the College and the Laboratories. Expenses for the Laboratories Operations are from private contributions.			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name A. Stanley Weltman, Ph.D.

Signature A. Stanley Weltman Date 6/25/74

Telephone 212 (Area Code) MA 2-1790 (Number) (Extension)

Checks payable to

Brooklyn College of Pharmacy

Mailing address for checks

600 Lafayette Ave., Brooklyn, N.Y. 11216

Responsible officer of institution

Typed Name Seymour Schertz

Title Comptroller

Signature Seymour Schertz Date 6/25/74

Telephone 212 (Area Code) MA 2-4040 (Number) (Extension)

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Item #7. Brief description of specific research aims (continued):

of treatment. Significant decreases in relative liver weights at 29 weeks in conjunction with decreased plasma cholesterol levels suggested the possibility of decreased hepatic cholesterol synthesis in the SHR.

Specifically effects of nicotine and/or cholesterol regimens will be investigated in spontaneously hypertensive and normotensive strains for a 1½ year period (Group I-nicotine; Group II-cholesterol; Group III-nicotine-cholesterol; Group IV-control). Aliquot populations will be sacrificed after 6 months of treatment. The present proposal has the following objectives:

1. Observation of weekly body weights and food consumption data would furnish information on effects of the treatments on body growth and development as well as influences on food metabolism and food utilization processes.
2. Similar measurements of water consumption (daily) and for the total week would be made to ensure intake of the required dose of nicotine/day as well as the comparative effects of the various regimens on water consumption and metabolism in the respective strains.
3. Comparison of base-line systolic blood pressure levels obtained prior to initiation of the treatment and at spaced intervals during the long range studies would determine time-sequential and progressive effects of nicotine and/or cholesterol on the systolic blood pressure levels of the respective groups. In addition effects of age on systolic blood pressure levels can likewise be ascertained.
4. Periodic sampling of blood specimens from the orbital sinus can be obtained for lipoprotein assays (electrophoresis). Time-sequential evaluations can be derived of not only the lipoproteins but also plasma cholesterol, triglyceride, phospholipid and FFA levels indicating the effects of the agents on the cholesterol and lipid profile as well as lipid metabolism. Relationships between cholesterol and lipid levels to the etiology of hypertension as well as peripheral and cardiovascular pathologies, etc. can be determined and evaluated.
5. Similar periodic sampling of orbital sinus specimens can be taken for plasma glucose and total plasma protein assays during the long range period.
6. To ascertain nicotine and nicotine metabolite levels (i.e., cotinine, etc.) as a function of the oral intake of 2.28 mg/kg/day of nicotine, assays will be undertaken using GLC (gas liquid chromotography) procedures to determine the nicotine blood levels, in the treated groups. At autopsy, liver specimens will be extracted for nicotine and metabolite assays.
7. Autopsy of aliquot populations at 6 months and 1½ years after initiation of treatment will demonstrate effects on endocrine and associated as well as vital organs. Thus, the adrenals, thymus, spleen, testes, seminal vesicles, liver, kidneys and hearts will be removed and weighed. The effects of the substances on such organs as the adrenals and gonads and their respective target organs will be evaluated. Similarly, heparinized blood samples obtained after decapitation can be assayed for the lipoprotein, cholesterol, triglyceride, phospholipid, FFA, glucose and total protein.
8. At autopsy, gross examination will be made of the brain, lungs, heart, kidneys, blood vessels etc for pathological observation. In addition, brain, heart, lung, kidneys, aorta, pulmonary artery, renal blood vessels, tissues and organs will be fixed in 10% formalin for appropriate histological evaluation of peripheral, cardiovascular and cerebrovascular arteriosclerotic and atherosclerotic abnormalties due to the respective treatments.
9. Thus, statistical analyses, comparisons and evaluation of blood pressure levels, plasma lipid profile with possible pathological changes after intermediate and chronic treatment will aid in clarifying the controversial aspects and differential hazards which either the single or combined agents may pose to the hypertensive and normotensive strains and possibly to man. Although it is dangerous to extrapolate animal findings to man, the hypertensive rats represent an animal model somewhat comparable to essential hypertension. To date, review of the literature has revealed no evidence wherein nicotine and/or cholesterol effects were simultaneously studied in spontaneously hypertensive rats.

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Item 7. continued:

Introduction and Background

In the past, the effects of smoking tobacco and its active constituent nicotine have been controversial. Smoking has been accused of being an etiological factor leading to hypertension, coronary heart disease, arteriosclerosis, atherosclerosis, etc. (1-15). It has been claimed that in heavy smokers there is increased mortality due to hypertension and coronary heart disease (6,12,15).

Although the etiology of hypertension, arteriosclerosis and atherosclerosis is complex and obscure and subject to much controversy, emphasis has been placed on the possible causative role of high cholesterol and fat diets in the development of these disease states. Correlations have been reported between abnormally elevated serum lipid levels and the incidence of ischemic cardiovascular disease. The striking association between serum cholesterol levels and atherosclerotic cardiovascular disease in the genetic disorder familial hypercholesterolemia has been well documented (16,17). Some types of hyperlipidemia are know to predispose subjects to coronary heart disease (18,19), peripheral vascular disease (20,21) and a possible relationship to cerebrovascular disease (21,22).

Since Fredrickson and Lees in 1965 (23) and in subsequent reports(24) proposed a system for phenotyping hyperlipoproteinemias, the concept of coronary disease detection and prevention using lipoprotein assays as a clinical tool has become exceedingly prevalent. Findings have suggested that if identified at an early stage coronary-prone individuals may be treated effectively to prevent premature disability and death (25,26). As a result of Fredrickson and Lees studies (23,24), the abberrant states of hyperlipoproteinemias have been classified into 5 types dependent upon variations in the proportions of chylomicrons, alpha lipoprotein, beta and prebeta lipoprotein fractions. It has been claimed that the beta fraction carries the major portion of cholesterol while the prebeta carries the major share of the endogenous triglycerides.

Previously, Wenzel et al (27) reported that 2.28 mg/kg of nicotine alkaloid administered orally to normal female rats and physiologically equivalent to the intake of "two packs of cigarettes per day" induced a biphasic effect on systolic blood pressure by first elevating it for 15 weeks followed by subsequent significant decreases in blood pressure upon continued oral administration. With larger oral doses (equivalent to 3 and 4 packs of cigarettes per day) only depressor effects on systolic blood pressure levels of anesthetized female rats were observed (28). Administration of either "low" or "high" doses of nicotine lowered the systolic pressure of renal hypertensive rats to below control levels (28).

Studies by our laboratory after oral administration of 2.28 mg/kg of nicotine alkaloid to spontaneously hypertensive male rats for six (29) and 29 (30) week periods indicated among other observations reductions in body weights, significant decreases in plasma cholesterol titers but no significant alterations in FFA and suggestive trends of reductions in systolic blood pressure levels. Significant decreases in the relative liver weights in conjunction with the decreases in plasma cholesterol suggested the possibility of decreased hepatic cholesterol synthesis in the 29 week study. Thus, as a consequence of the diverse reports of higher cholesterol levels in smokers (1,2), this aspect as well as detailed assays of the plasma lipid profile warranted further investigation.

In view of reports that smoking caused a larger rise in blood pressure levels of hypertensive subjects than in normal subjects (3) and administration of "low" or "high" doses of nicotine to renal hypertensive rats lowered blood pressure to below control levels (28), our initial quest had been to determine possible hypertensive and/or hypotensive effects of nicotine in the spontaneously hypertensive rats. Indications of reductions in systolic blood pressure levels appeared to suggest that the spontaneously hypertensive rats like like renal-hypertensive rats (28) may possibly be more susceptible to sympathetic ganglionic blockade by nicotine.

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Investigations by our laboratory of the acute effects (29,31) of subcutaneous administration of nicotine alkaloid (0.5 or 1.0 mg/kg) to spontaneously hypertensive male rats sacrifieed at 30 and 60 minute periods indicated that at 30 minutes, nicotine stress via increased adrenal catecholamine release induced mobilization of FFA in addition to elevations in plasma corticosterone. Nicotine-induced increases in cholesterol and hyperglycemia were observed at 60 minutes at which time plasma and adrenal corticosterone levels were decreased. The degree of adrenal depletion of corticosterone produced by acute nicotine stress in the spontaneously hypertensive rats suggested a lower rate of adrenal corticosteroidogenesis.

After 6 and 29 weeks of oral administration of nicotine alkaloid (2.28 mg/kg/day) to the SHR, no significant alterations were noted in either the plasma corticosterone, adrenal corticosterone or adrenal catecholamine concentration. Relative adrenal weights were significantly heavier in the nicotine-treated rats at 29 weeks.

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In our previous progress report/number 3 (June 1973) where nicotine alkaloid was administered orally to normotensive Wistar rats (CFW, Carworth, Inc.) not genetically related to the Japanese spontaneously hypertensive strain, similar significant decreases were noted in the body weights and significant increases in the relative adrenal weights at 29 weeks. Although relative liver weights and plasma cholesterol levels were decreased, these changes were not significant. Trends of slightly lower systolic blood pressure levels were also observed in the Carworth nicotine-treated normotensive rats after the 11th week, but the changes were not significant. At 29 weeks, significant decreases were observed in the plasma glucose levels of the nicotine-treated normotensive rats. Smaller decreases in the glucose levels of the nicotine-treated hypertensive rats were not significant. One may question the contributing effects of the differences in the genetic origin of the Japanese and Carworth strains on the response patterns of the hypertensive and normotensive rats to nicotine. The present proposal involving investigations of the SHR and WKY/N strains will minimize genetic diversity since the SHR and the normotensive WKY/N stocks have the same genetic origin.

involved

Additional aspects of the various nicotine investigations/joint collaborative efforts with Drs. Valentin Yermakov and Stefan Schwan, staff members of the Dept. of Pathology at Downstate Medical Center of New York. The objectives were to determine possible increases in peripheral, cardiovascular and cerebrovascular pathology due to nicotine administration in the spontaneously hypertensive rats. To date, continuing histological studies have revealed no evidence of an increase in peripheral, cardiovascular and cerebrovascular pathology which could be attributed to nicotine in the spontaneously hypertensive rats (administration of nicotine for 29 weeks; other studies under examination).

Smoking and Body Weights

In terms of smoking effects on body weights, Damon (2) reported lean men smoked significantly more than fat subjects. Karvonen et al (1) reported smokers of rural regions were slightly thinner whereas urban smokers showed no difference in body weights between smokers and nonsmokers. In the Thomas (6) investigations which represented an urban group of medical students, smokers have a larger proportion of heavy individuals. In contrast Brozek and Keys (32) reported that men who stopped smoking tended to become fatter. Recent studies (33) have similarly indicated that smokers have lower body weights than nonsmokers and that ex-cigarette smokers gained more weight and were heavier than smokers and nonsmokers.

Smoking and/or Nicotine Blood-pressure Studies

It is quite evident from smoking studies in man (7,34) and from animal investigations (35,36) that acute tobacco smoke and/or nicotine can produce transient increases in blood pressure, etc. Whether the habit of smoking tobacco is related to the development of hypertension, coronary heart disease and arteriosclerosis has long been the

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subject for discussion (2,6,11,12). Yet, in epidemiological studies to correlate cardiovascular changes with smoking, Hadley (14) reported that the average blood pressure of smokers was somewhat less than non-smokers. Hammond and Horn (15) and Damon (2) have also been unable to establish a relation between cigarette smoking and hypertension. Blackburn et al (4) reported distinct tendencies to lower systolic and diastolic blood pressures in chronic smokers but found higher basal and resting pulse rates in smokers. It has also been reported that after smoking 2 cigarettes, habitual smokers show rather prolonged pressure responses and longer sustained increase in heart rates than do non-smokers (10). Thomas (6) further compared characteristics of smokers and non-smokers with family histories and claimed that student smokers presented family histories of parental hypertension more often and had higher mean recumbent values for heart rate and pulse pressure, but noted that student non-smokers had higher mean recumbent values for diastolic pressure. Smoking has also been reported to cause a larger rise in the blood pressure levels of hypertensive subjects (3) than in normal subjects. Recent findings reported by Seltzer in 1974 (33) claimed that cigarette smoking tended to have an inhibitory effect on blood pressure levels. Cigarette smokers presented lower, over-all mean systolic and diastolic blood pressure levels than non-smokers or quitters.

Chronic studies with animals involving effects of nicotine on blood pressure have also been inconsistent. In part, these can possibly be related to dosage, mode of administration, species, etc. Haag et al (36) exposed rats to chronic cigarette smoke for periods up to 2 years and reported lower blood pressure levels that were not statistically significant and no evidence of chronic hypertension. When the blood pressures were determined prior to and after exposure to cigarette smoke, significant increases were noted in the systolic blood pressure which persisted for 45 minutes. In contrast, rabbits given nicotine alkaloid in drinking water, revealed significant and cumulative increases in systolic blood pressure during a 24 week period (37). Similarly, Bhagat (38) administered nicotine subcutaneously to rats daily for 6 weeks and Westfall (39) for 8 weeks; both demonstrated gradual and significant increases in systolic blood pressure as administration continued. Paradoxically, Wenzel (27) also adminstered nicotine in drinking water to rats and reported significant, cumulative elevations in tail systolic blood pressure up to and through the 15th to 20th weeks, followed by a gradual decline to normal levels at week 30 and significantly lower blood pressure readings at week 55. A similar biphasic effect on blood pressure by nicotine had previously been reported by Kin (40) wherein initial elevations of blood pressure of rabbits were followed by a secondary lowering to below control levels as the treatment with the drug was prolonged. These paradoxical observations of increases in blood pressure of animals followed by decreased levels in the same animals as treatment continued along with similar reports of somewhat lower blood pressure levels in chronic smokers pose interesting questions. Conceivably, these decreases may result from chronic sympathetic blockade or depletion of vascular tissue norepinephrine by nicotine. Similarly, the questions of the development of tolerance and/or facilitation must also be considered. In studies of the reaction of chronic smokers, Roth and Schick (3) reported that habitual smokers did not show tolerance to the immediate effects of smoking as indicated by blood pressure, pulse rate and skin temperature measurements which involved 66 standard smoking tests. In contrast, Thienes (41) reported that rats subjected to pivoting response tests showed the development of tolerance within 2 weeks, no tolerance to convulsive and fatal doses of nicotine and some indications of tolerance development by the adrenal medulla to the stimulating effects of nicotine.

In doses absorbed by cigarette smokers during and shortly after smoking, nicotine the active constituent, has been found to increase heart rate, raise arterial pressure dilate arterial blood vessels of muscles while contracting those of the skin, to increase cardiac output (10) and to reduce the skin temperature of the extremities, etc.

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(34). Thus nicotine produces a complex array of cardiovascular responses and hemodynamic effects in which the precise mechanisms cannot be readily defined (42). The low dose effects of nicotine have been ascribed to the release of catecholamines from the adrenergic nerve endings of the adrenal medulla (42,43), with stimulation of the sympathetic ganglia, etc. (42). In large doses, nicotine blocks ganglionic transmission and can paralyze ganglion cells. By virtue of its ability to stimulate the parasympathetic nervous system, nicotine can elicit responses opposite to sympathetic stimulation (44,45). The parasympathetic ganglia require higher concentrations than the sympathetic ganglia for direct stimulation (44). Similarly, other diverse and opposing mechanisms can occur, in that while stimulation of the aortic and carotid chemoreceptors causes increases in arterial pressure, heart rate, respiration rates, etc., activation of the pulmonary and coronary artery receptors produce bradycardia, hypotension and apnea(44,45). It is important to note that nicotine action varies with species, as in the cat. Vasoconstriction by nicotine has also been induced by stimulation of the posterior pituitary to secrete antidiuretic hormone and by direct action of nicotine in the blood vessel walls of the arteries (3).

Smoking and Cholesterol, etc.

Again conflicting reports have been cited regarding the relationship of chronic smoking habits with blood cholesterol levels. Whereas, Blackburn et al (4) did not observe any significant differences between cholesterol levels of smokers vs nonsmokers, others have reported positive correlationships and significantly higher blood cholesterol levels in smokers (1,2,6,46). Acute effects in smokers after 2 cigarettes were found to show significant increases in free fatty acids and blood glucose levels but no significant alterations in the blood cholesterol and triglyceride levels (47).

In rabbits, elevated plasma cholesterol levels have been obtained after continued oral administration of nicotine and similar effects after nicotine injections (48,49). Injection of nicotine in dogs for 6 weeks was found to significantly elevate serum cholesterol, although no significant change was observed in serum triglycerides (9). It was suggested that the cholesterol increases were secondary to nicotine induced release and rise of free fatty acids which stimulated increased cholesterol synthesis by the liver. Acute responses of dogs to inhalation effects of cigarette, pipe, cigar smoke, etc. have demonstrated mobilization of serum FFA as well as increased triglyceride levels (50).

Smoking and Adrenal Activity

The important role of the adrenal gland and the sympathetic nervous system in control of free fatty acid liberation has been well recognized (51,52). Smoking in man and administration of nicotine to dogs have likewise demonstrated rapid mobilization of free fatty acids from the fat depots due to stimulated adrenal medullary activity and increased catecholamine secretion (53). Tobacco smoke or nicotine administered to cats have similarly demonstrated stimulated release of catecholamines from the adrenal medulla (35).

Adrenocortical stimulation in man by tobacco smoke and also in dogs and rats by acute nicotine administration have likewise been reported (54). It was suggested that the increases in corticosteroid levels resulted secondarily from nicotine-induced catecholamine activity enhancing corticotrophin release. Regarding thyroid function, Blackburn et al(4) indicated no significant difference in blood PBI values of smoker vs nonsmoker subjects.

Nicotine, Cholesterol and Atherosclerosis

In studies of atherogenic effects of cholesterol diets, increases in atherosclerosis have been reported in rabbits treated with nicotine (49,55). Stefanovich et al (56) further reported that 2.28 mg/kg of nicotine administered orally to rabbits fed an

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atherogenic diet increased the degree of aortic atherosclerotic lesions as well as serum cholesterol levels in rabbits at 6 weeks. Wenzel et al (57) however, in rabbits administered nicotine orally at dose levels of 1.14 mg/kg observed no histological and pathological differences between groups given nicotine and an atherogenic diet or the diet alone. This may be a reflection, however, of the lower dose of nicotine which was equivalent to 1 pack of cigarettes per day. It is of interest that recent studies comparing blood cholesterol levels in smokers, nonsmokers and subjects eating 0,1, and 3 eggs/day revealed that the serum cholesterol response to egg intake was amplified by smoking (58).

Chronic Effects of Nicotine

In chronic studies with animals, some investigators have reported harmful effects of chronic nicotine poisoning such as retardation of growth (59-62), adrenal enlargement or injury (59,63,65), interference with reproduction (59,66,67) and atrophy of the gonads (59,63,68). Many of these findings were based on studies of small animal population. Others, exposing rats to tobacco smoke (36) reported no significant differences in blood pressure, longevity, reproduction and pathology.

Cholesterol diets and and atherosclerosis

Cholesterol diets have produced atherosclerosis in rabbits (69,70) and have been reported as early as 1934 (69). Dauber and Katz (71,72) were the first to show that atherosclerosis could be consistently produced in the chicken by feeding diets high in cholesterol. A correlationship was further demonstrated between the degree of atheromatosis development in the chicken and the amount of cholesterol added to the diet (73). Cockrels fed an atherogenic diet demonstrated aortic atherosclerosis as well as elevated plasma cholesterol levels at 27 weeks (74). In rats, the production of cardiac thrombi and infarcts have been induced by the ingestion of thrombogenic diets (high fat-cholesterol diets containing in addition propylthiouracil) (75,77). Other studies have indicated that the degree of spontaneous arteriosclerosis produced by repeated breeding of a strain of male and female rats (78) was augmented by a high fat-cholesterol diet and/or ACTH administration (79). Investigations with cholesterol-fed rats have likewise demonstrated hyperlipoproteinemia with altered distribution in in the subunit composition of the serum lipoprotein fractions (80). Certain beagle dogs displaying marked endogenous hyperlipoproteinemia and atherosclerosis even when fed low fat commercial diets were found to be hypothyroid. Admininstration of dessicated thyroid decreased serum cholesterol, triglycerides and lipid concentration affecting lipoprotein fractions at density <1.060 (81). Thyroid hormones in man have also been reported to decrease plasma cholesterol while increasing cholesterol biosynthesis and catabolism (82).

Hypertension

In elucidating the etiology and pathogenesis of essential hypertension in man, various mechanisms or factors have been causally related to heightened blood pressure. Thus, the influence of neurogenic and psychologic factors have been widely recognized (83-85) as well as various hormonal factors (86-97). In man, hypertension has been variously related to primary aldosteronism (86-88), adrenal adenomas and hyperplasia (84,89), Cushing's Syndrome (83,90), etc., all resulting in increased adrenocortical secretions. Pheochromocytoma or tumors of the adrenal medulla wherein large amounts of epinephrine and norepinephrine are secreted (91) and norepinephrine have similarly been implicated in the pathogenesis of human and experimental hypertension (92). Excessive secretions by the thyroids (Graves disease) have likewise been associated and found to induce hypertension in man (93). Conversely, it is of interest that antithyroid treatment in rats provided significant protection against full development of renal hypertensions (94). The renin-angiotensin system has also been involved in hypertension and regulation of blood pressure (88,95-97).

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Spontaneously Hypertensive Rats

In 1963 (98), a strain of spontaneously hypertensive rats (SHR) was selectively bred which has been considered most appropriate for studies relating to essential hypertension. Extirpative procedures as well as exogenous hormones administration have been used to demonstrate the active role played by the pituitary-adrenal and pituitary-thyroidal axis in inducing and maintaining the hypertensive state in the SHR strain (99-101). Other investigations have demonstrated a similar contributory role of the adrenal-medulla and increased catecholamine output with the development of the spontaneously hypertensive state (102-104). Studies with the hypertensive strain have similarly indicated that the severity of hypertension can be modified and accentuated by gross increases in Na and salt intake (104). No apparent increased activity has been noted in the renin-angiotensin system of the spontaneously hypertensive strain by some investigators (105). Recent studies (106-108), however have reported hypothyroidal function rather than hyperthyroidism(99-101) in the spontaneously hypertensive rats.

The spontaneously hypertensive rats have been found to develop hypertensive states exceeding 150 mm. Hg, within 2 months to 15 weeks with an incidence of 100%. The degree of hypertension has been found to increase with age and to reach levels as high as 200 mm Hg and over (98-101,103,109). The absence of abnormalities in the kidneys except at the terminal stages of hypertension (109) accompanied by the spontaneous nature and the endocrine resemblances have thus caused the investigators to consider this strain an excellent model for studies of essential hypertension(98, 101,109).

Analyses of the pathological changes in the blood vessels, heart (cardiac hypertrophy, etc.), kidneys and brain of the spontaneously hypertensive rats (109) have paralleled changes found in the cardiovascular pathology caused by essential hypertension in man. Similarly, pathological and histological changes noted in the pituitary, adrenals and thyroids of the SHR strain (110) have also paralleled endocrine and tissue changes correlated with hypertension in man. To illustrate, hypertrophy and "hyaline changes" were noted in the pituitary of the hypertensive rats, as well as hypertrophy of the glomerular and fascicular zones of the adrenals, etc. Augmented cerebrovascular (111) and cardiovascular (112) pathologies have been demonstrated in spontaneously hypertensive rats give 1% saline for drinking water with and without a high fat-cholesterol diet.

Spontaneously Hypertensive Rats and Cholesterol Diets

Umehara et al, (113) observing effects of high-fat diets, reported elevated total blood cholesterol values in spontaneously hypertensive rats as well as degenerative lesions in the peripheral arteries and arteriosclerotic changes in the arteries of the brain, heart and kidneys. In contrast, Nagaoka et al (114) claimed that feeding a high cholesterol diet (2%) plus 0.15% propylthiouracil or a diet of 2% cholesterol to spontaneously hypertensive rats demonstrated that cholesterol was not involved in the progression of hypertension nor in the occurrence of thrombus formation. Feeding of the combined cholesterol and propylthiouracil diet however, consistently elevated plasma cholesterol levels above control diet values. Plasma cholesterol levels of animals fed cholesterol alone were lower than the cholesterol - PTU group, but higher than in the spontaneously hypertensive rats fed the control diet. Studies involving diets containing 1% Na Cl or 1% Na Cl plus high-fat and cholesterol levels were reported to significantly induce fibrinoid necrosis of arterioles and arteries and increase pathology in the kidneys, hearts and brains of spontaneously hypertensive rats (112) as well as produce a high incidence of cerebrovascular diseases, infarcts and hemorrhages in the SHR (111).

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The present investigators have published investigations with hallucinogens such as lysergic acid diethylamide (115-119) and mescaline (120-124) on the metabolism behavior and endocrine function of rats and mice.

Dr. Weltman has also engaged in studies related to the effects of auditory stress (125-127), vibration stress (128-130), isolation stress (131-135) as well as behavioral, metabolic and physiological differences in audiogenic-seizure susceptible vs.resistant rats (136-138) and the excitable homozygous-whirler vs. normal, heterozygous-whirler mutant mice (139-145). The various behavioral, biochemical and endocrine studies have indicated heightened metabolism rates, increased adrenocortical function and, in general, inhibited gonadal activity in the whirler mice. These a may be symptomatic and correlated with physiological and neuronal changes responsible for the wild, circling, locomotor activity. Biochemical alterations have indicated significantly increased plasma corticosterone (142,143), adrenal corticosterone (142,143) and adrenal catecholamine levels (143) accompanied by significant alterations in carbohydrate metabolism (142,143,145) (i.e., lower plasma glucose and liver glycogen levels). Although total plasma protein levels were significantly reduced due to depressions in $\alpha 1, \alpha 2$, beta and gamma globulins, albumin levels were significantly higher (142).

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Item #8. Brief statement of working hypothesis (continued):

Similarly, while many investigators associate tobacco smoking with hypertension and coronary heart disease, epidemiological studies (33) have indicated that the average blood pressure levels were somewhat lower in smokers than nonsmokers. On the other hand, smoking has been reported to cause larger rises in blood pressures of hypertensive subjects than in normal subjects (3).

Our findings involving effects of 6 and 29 weeks of oral nicotine administration (2.28 mg/kg/day, equivalent to two packs of cigarettes per day) revealed significantly decreased total cholesterol levels in male spontaneously hypertensive rats and showed indications of somewhat lower blood pressure levels in the nicotine-treated or untreated SHR. Nicotine has also been found to lower the blood pressure levels of renal hypertensive rats (28).

While the present investigation is not devised to demonstrate mechanisms affecting cholesterol metabolism in the SHR, the detailed time-sequential studies involving blood lipoprotein, cholesterol, triglycerides, phospholipid and FFA will yield a clearer understanding of the effects of nicotine on cholesterol and lipid metabolism in the spontaneously hypertensive rats. Similarly, the intensive study should demonstrate possible synergistic, antagonistic etc. interaction and relationships of nicotine and/or cholesterol intake with hypertension, cholesterol and lipid metabolism and the possible development of vascular, arteriosclerotic and atherosclerotic pathologies. Comparable studies with the genetically related normotensive strain will indicate a measure of the intermediate and prolonged effects which nicotine and cholesterol pose to initially normotensive individuals as well as possible diversities in the response patterns of the hypertensive and normotensive strains to nicotine and/or cholesterol intake. There are no indications that investigations have been undertaken to compare and clarify effects of simultaneous nicotine and cholesterol administration on blood pressure, lipid profile and vascular pathology in spontaneously hypertensive rats.

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Item #9. Details of experimental design and procedures (continued):

of nicotine. Records will be maintained of the volumes consumed during the 18 hour period and plain tap water during the remaining 6 hour period. The 2.28 mg/kg/day dose has been reported to have the psychic equivalence of the daily intake of "two packs of cigarettes per day" (48). Since 4 rats will be caged in a unit, Group A animals will receive an average rather than an exact daily dose which they would have received if they were injected. The oral dose and method, although not exact, is preferable since it simulates the gradual daily smoking of cigarettes; it avoids the stress of too frequent injections and the possibility of convulsive doses being administered via the injection procedures. The nicotine alkaloid will be obtained from Eastman-Kodak.

Group B animals will be supplied a cholesterol enriched diet mix consisting of 5% cholesterol and 95% Purina Laboratory Chow Meal. To insure even distribution of the cholesterol, the ingredients will first be mixed in a mortar followed by agitation in a container on a horizontal shaker until uniformly dispersed (20 min.).

Group C animals will receive equivalent oral doses of nicotine and the cholesterol-diet mix furnished to Group B. Group D will represent the untreated control spontaneously hypertensive group. In all cases, the procedure for changing bottles containing nicotine and/or water alone will be followed in all groups to make sure that all cages and groups receive identical handling or disturbances.

The following schematic outline will represent the population sizes for the Series I (spontaneously hypertensive rats) and Series II (normotensive rats) studies:

Series I - - SHR
Group 1 - - Six months
Group A - nicotine group (dose 2.28 mg/kg/day) - 16 rats
Group B - cholesterol enriched diet mix (5%) - 16 rats
Group C - nicotine (2.28 mg/kg/day); cholesterol diet (5%) - 16 rats
Group D - control - tap water - 16 rats
Group 2 - - One 1/2 years
Group A - nicotine - 20 rats
Group B - cholesterol diet - 20 rats
Group C - nicotine-cholesterol diet - 20 rats
Group D - control

Group 1 will contain a total of 64 rats and Group 2 of Series I a total of 80 rats consisting of 144 rats. The longer chronic study will consist of a larger population in view of the possibility of premature deaths.

Group 1 (6 months) and Group 2 (1½years) of the Series II study (normotensive rats, WKY/N) will have the same schematic outline and population sizes. Thus, a total population of approximately 288 animals will be used in the present proposal.

During the prolonged 1½ year chronic investigations heparinized blood samples will be collected from the orbital sinus (147) at the end of 2,6,12,18,24,32,40,52 and 64 weeks of treatment. Plasma samples will be assayed for total cholesterol (148), triglycerides (149), phospholipids (150), glucose (151) and total plasma protein (152) titers. Lipoprotein assays (153) will be determined by use of a Helena Laboratories electrophoretic equipment and densitometer (Electrophoresis Chamber and Quick Scan Densitometer, Model 1111). The preceding analyses will be determined on an individual basis since small plasma volumes are required for the respective analyses. FFA (154) determinations will be derived by pooling the remaining blood of 3 plasma samples for one FFA assay due to the larger volume of plasma required for the FFA quantitative procedures.

At appropriate intervals, heparinized plasma samples will be assayed for nicotine and metabolite titers (i.e., cotinine etc.) using a Perkins Elmer Model 811 gas chromatograph equipped with an hydrogen flame ionization detector and a Speedomax G recorder (155). At time of sacrifice, liver specimens will likewise be assayed for nicotine and metabolite levels. These tests will assist in estimating and correlating the level of nicotine intake

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with the effects of nicotine on the various parameters in the nicotine and nicotine-cholesterol-treated rats.

To determine the effects on endocrine organs, as well as possible pathological alterations due to the nicotine and/or cholesterol regimen, aliquot and representative populations of the spontaneously hypertensive and normotensive group will be sacrificed after 24 weeks of treatment. Heparinized plasma samples will be collected following rapid decapitation with a Harvard decapitator. Similarly, at the end of the 1½ year testing period (78 weeks), the remaining groups will be similarly decapitated for plasma collections, organ weight as well as gross and subsequent histological evaluations of pathological alterations.

Systolic blood pressure measurements will be obtained from the respective tests and control groups prior to initiation of the agents (0 weeks) and following 4,8,16, 23,30,38,50,62 and 77 weeks of treatment. Animals to be sacrificed will have their blood pressures determined 1 week prior to sacrifice to reduce the possibilities of stress effects on biochemical parameters.

Upon sacrifice at the 24 and 78 week periods, the animals will be rapidly dissected for observation of effects of nicotine and/or cholesterol on adrenals, thymus, spleen, liver, testes, seminal vesicles, kidneys, heart and brain weights. Decreases or involution of the thymus and spleen lymphoid organs have been used as a measure of increased adrenocortical and steroidal output. Alterations in the testes and seminal vesicle weights will reflect gonadal influences by the respective treatment. The body cavities, brain, lungs, kidneys, heart and vascular system will be examined for gross pathological abnormalties resulting from hypertensive, arteriosclerotic and atherosclerotic alterations. Tissues and organs such as the heart, kidneys, pulmonary artery, aorta, renal blood vessels, brain, lungs etc. will be fixed in 10% formalin solution for histological and microscopic evaluations of arteriosclerotic and atherosclerotic pathological anomalies. Among other factors animals will be examined for cardiac infarctions (scarring and hypertrophy) periarteritis nodosa, nephrosclerosis, cerebral hemorrhage, lung involvements, etc.

* Note:

The present proposal does not include in the budget the costs for histological processing of the tissues and organs. As indicated, at the conclusion of the 6 month and subsequent 1½ year studies, the tissues will be fixed in 10% formalin. If biochemical, blood pressure, organ weight and autopsy data then indicate the merit for further histological examination, this phase will be pursued subject to the consent of the Council for Tobacco Research and a supplemental budget request to be submitted for this histological work.

In all series and groups, records be maintained of premature deaths. Whenever possible animals will be autopsied and examined grossly for pathological abnormalties and probable cause of death. Tissues and organs will be selected for histological examination when periods between time of death and final autopsy are not prolonged.

The various data will be analyzed by standard t-test procedures and analyses of variance (156) to determine significant effects of nicotine and/or cholesterol treatments in the spontaneously hypertensive and normotensive groups. Thus, body weight, food consumption, water consumption, food utilization ratios, blood pressure, organ weights and the various biochemical parameters will be compared by these procedures. Correlation procedures (156) will also be used to analyze i.e. cholesterol and blood pressure levels etc. and to determine direct or inverse relationships of the various biochemical parameters due to nicotine and/or cholesterol administration.

The laboratory has available a Cogito 566 P R model calculator(Marchant) as

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well as Friden 130 Electronic Calculators for computation of the data. In addition, the facilities of the Long Island University Brooklyn Center, Computer Center are available. The Computer Center has an IBM Model #1130-3 C computer and accessories for statistical analyses involving variance, correlations etc. The services of Mr. Anthony Brogna, Director of the Computer Center and the equipment are consequently available to us.

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Item #10. Space and facilities available (continued):

assays, a Perkins Elmer Model 811 Gas Chromatograph with a hydrogen flame ionization detector and a Speedomax G recorder is available for nicotine and nicotine metabolite assays. Helena Laboratories Inc. Electrophoretic and Densitometer equipment (Electrophoretic Chamber and Quick Scan Densitometer, Model 1111) are available for the lipoprotein assays.

The animals are housed in an air-conditioned room approximately 22' x 27' (594 sq. ft.), provided with an exhaust system, which can contain 8-9 animal racks. Cages for mice or rats are available depending upon the particular study. The animal room contains water facilities and a drainage system for proper sanitary needs. Adjacent to the animal room are 2 storage rooms approximately 5' x 10' (50 sq. ft.) and 5' x 13' (65sq. ft.). These are used to store food, shavings and other sundry supplies. Also adjacent to the animal quarters is a behavioral study room 7' x 12' (84 sq. ft.) used for O_2 consumption, locomotor activity and blood pressure studies. This room permits animals to be observed and studied in relative quiet. A separate room 10' x 17' (170 sq. ft.) removed from the animal room by a corridor and 2 doors serves as office space and area for auditory stress studies. This separation prevents extraneous noise from bells, etc., to reach and disturb animals in the animal quarters. A washroom, approximately 12' x 17' (204 sq. ft.) contains an automatic-spray washing machine and sinks which are used to sterilize and cleanse cages and water bottles. The main research laboratory approximately 16' x 50' (800 sq. ft.) is provided with desks, table tops, cabinets and much of the equipment cited above. This room contains 3 water-sinks and is the area where autopsies, hematological, histological, and biochemical tests are performed and where calculations are done.

b) Institute of Pathology
Downstate Medical Center, S. U. N. Y.
450 Clarkson Avenue
Brooklyn, N. Y.

At the Institute of Pathology, laboratory rooms and equipment are available for sectioning and automatic fixing and staining of the preparations and slides. They consist of microtomes, auto-technicons, microscopic equipment, glassware and accessory supplies. An electron microscope and fluorscent apparatus are available if these techniques are needed.

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

May 16, 1974

Grant application No. 983

PHARMACOLOGY

To: The committee comprising Drs. Gardner, Meier and Sommers

Subject: Leo G. Abood, Ph.D., University of Rochester Medical Center
New application No. 983
"Behavioral effects of Nicotine and Piperidine using a Computer-Controlled Program"

History

A preliminary inquiry was handled as case No. 261 and the Executive Committee voted to encourage formal application.

Request

Application No. 983 requests $20,125 plus two additional years.

Documents Submitted

Attached is undated application received by CTR May 10, 1974.

Reprints or preprints of the papers listed on page 12 were provided, and will be forwarded to you promptly if you wish.

Comment

We shall seek an appropriate outside reviewer to evaluate this proposal.

FWN:gh
Enclosure

F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

MAY 10 1974

Application for Research Grant

(Use extra pages as needed)

Date:

1. Principal Investigator (give title and degrees):
   L.G. Abood, Professor of Biochemistry and Brain Research, Ph.D.
   K. Lowy, Professor of Brain Research, M.D. (co-principal investigator)

2. Institution & address:
   University of Rochester Medical Center
   Rochester, New York 14642

3. Department(s) where research will be done or collaboration provided:
   Center for Brain Research

4. Short title of study:
   Behavioral effects of nicotine and piperidine using a computer-controlled program.

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: December 31, 1977

7. Brief description of specific research aims:

The overall objective of this program is to determine the behavioral effects of nicotine and piperidine in cats with a view towards a better understanding of the involvement of cholinergic systems in specific behavioral parameters.

1) Determine the effect of both nicotine and piperidine on a number of quantifiable psychophysical parameters associated with computer-controlled behavioral paradigm in cats.

2) Determine the extent to which nicotine and piperidine will reverse the alterations in the psychophysical patterns produced by an anticholinergic psychotomimetic glycolate, N-methyl 4-piperidyl cyclobutylphenyl glycolate.

3) If some distinct psychophysical parameters are observed with nicotine, conversely, we plan to test a variety of glycolate esters for their ability to reverse such effects.

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8. Brief statement of working hypothesis:

Our hypothesis is that there are poorly understood nicotinic systems within the brain that are involved in many aspects of brain function. With the use of nicotinic drugs and their antagonists and making use of more sophisticated techniques for measuring behavior we hope to determine certain behavioral manifestations of such systems. Of particular significance is the fact that piperidine, a known nicotinic drug, is an effective tranquilizer and, possibly, an endogenous psychochemical or neurotransmitter.

9. Details of experimental design and procedures (append extra pages as necessary)

Background

As part of an extensive program to investigate the structure-activity relationships and mechanisms of actions of a group of anticholinergic psychotomimetic glycolate esters (1,2) it was observed that certain cholinergic agents acted as effective antagonists to the agents. Among the most effective agents in this group were physostigmine, tetrahydroaminoacridine, and piperidine (1,3). All three, which were nicotinic as well as muscarinic agents, reversed both the peripheral anticholinergic and psychotomimetic actions of the glycolate esters. The most interesting of the three was piperidine because of its extremely low toxicity and since it was found to have a pronounced tranquilizing effect in animals and man (4).

An extensive clinical trial with piperidine was then undertaken to determine its efficacy as a tranquilizing drug. It was first tested in a large group of psychotic patients (2-4 g/day orally) in a maximal security division of a state hospital, and the results were quite dramatic (4). Unfortunately when tested on another group of psychotic patients at the same hospital and elsewhere, the drug had to be discontinued because of severe nausea and vomiting in 1/3 of the patients. It was later determined that 75% of the first group of patients (maximal security) were heavy smokers and, consequently, had developed a considerable tolerance to the nicotinic action of piperidine. Nevertheless, because of this serious side effect, interest in piperidine as a potential psychotherapeutic agent rapidly subsided.

There are, however, other reasons for an interest in piperidine. Its presence in mammalian brains has been known for some time (5,6), and, recently, has stimulated more interest (7,9). Dolezalwa et al (9) have demonstrated that piperidine may have a physiological role in the molluscan central nervous system, insofar as endogenous piperidine tends to accumulate in the central ganglia of snails during hibernation. They also showed that minute amounts (10^{-14} moles) injected microionphoretically into the ganglionic neurons mimicked the threshold inhibitory

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effect of acetylcholine on some cells and had a cholinolytic action on the other cells. Such responses are reminiscent of the action of the nicotinic-muscarinic action of acetylcholine in peripheral cholinergic systems.

Previous work done by us

We have been interested in developing psychophysical measurements which may provide more accurate quantitative and qualitative evaluation of more complex behavioral patterns that may assist in the evaluation of various psychotomimetic and other psychotropic drugs. An anticholinergic psychotomimetic agent was examined for its behavioral effects on cats trained to press a lever whose location corresponded to one of two sound sources. The cats were trained to lick a protruding sponge in dim light which then caused the main light to turn on and an auditory signal to be emitted from either side of a panel in the chamber. Any lever response terminated the trial. A food reward was given only if the cat pressed a lever on the same side as the sound signal. A new trial cycle began when the cat licked the sponge. A computer program controlled the experiment, stored the experimental data, and permitted an analysis of various psychophysical parameters, such as ability to localize an auditory cue, threshold of sound intensity, rate of trial onset, and lateral tendency. Doses of 10 - 20 µg/kg, N-methyl 4-piperidylcyclobutylphenyl glycolate (CBG) reduced the number of responses, and tended to lower the relative time spent in the light period. However, at lower doses CBG produced a marked increase in some cats in total number of trials. Higher doses of scopolamine also reduced total trials, but less consistently. An increase of the number of responses was not observed. A number of animals exhibited a lateral preference for either the right or left lever. CBG, but not scopolamine, markedly shifted this lateral tendency in some cats. On the basis of these studies a number of correlations could be made with respect to the central effects of the glycolate esters in man.

Proposed Research Program

Our first objective is to determine the effects of nicotine and piperidine on cats, utilizing the computer-controlled program used in a previous study (16), and to be described in detail below. After a baseline has been established with a number of quantifiable psychophysical parameters, an attempt will be made to revise or block any effects with the centrally active anticholinergic glycolate esters. The converse study will also be undertaken: nicotine and piperidine will be used to reverse or block the behavioral effects of the anticholinergic glycolate esters.

Detailed description of behavioral program: A scheme of the behavioral program with the sequence of events is presented in figure 1. The sound-proofed testing chamber contained omnidirectional levers on either side of a sponge, all three being located in such a fashion that the cat could readily nudge the sponge with its nose from a standard position and subsequently press either of 2 levers. A schematic diagram (front) of the sound-proofed chamber showing the relative positions of the speakers, levers, and sponge is presented in figure 2.

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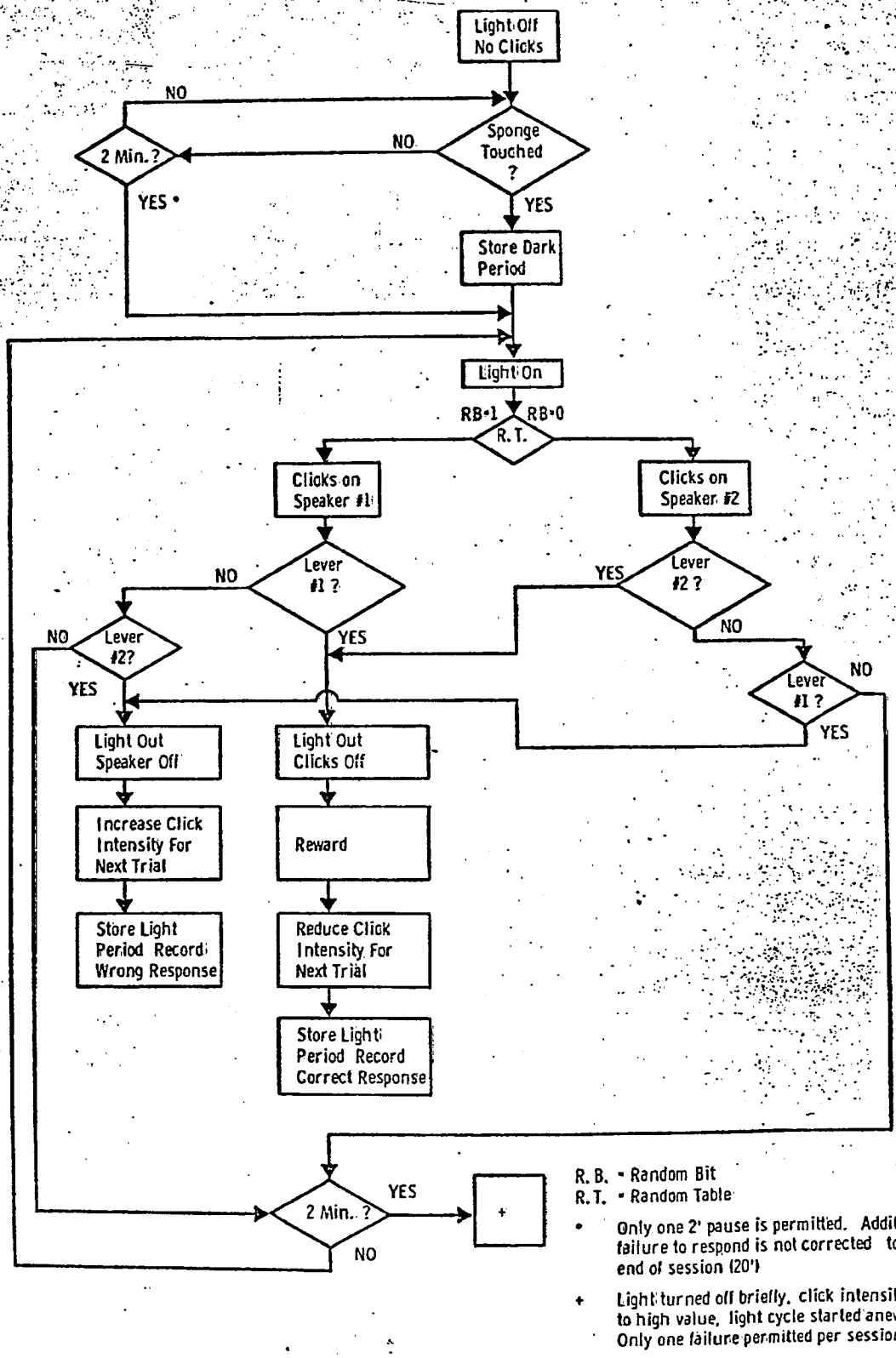

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Figure 1

Detailed scheme of behavioral program controlled by LINC 8 Computer

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The sequence of events, sound intensity, and sound source were controlled by a LINC-8 computer. The data, which were stored on magnetic tape, included the sequence and intensity of left and right clicks, the correct and wrong responses, and the duration of the light or dark periods. In addition, the tape also contained all necessary bookkeeping information: identification of animal, date, type and dosage of drug, as well as information of specific interest (e.g., erratic performance and pupillary reaction).

A single trial consisted of two phases. During the dark period (with only a small pilot light illuminating the feeding area), no click stimulation was presented, and the levers were inoperative. To start the light period, the cat had to touch a centrally located sponge connected to a contact-sensing device (drinkometer). This maneuver turned on the ceiling light and started a click series on one of the randomly selected speakers. The light period was terminated by the cat's touching one of the levers, which turned off the ceiling light and the clicks. Only a correct (homolateral to the speaker) response was rewarded by a small cup of milk. The animal consumed the reward during the dark period.

The initial training in selecting left versus right lever in response to loud clicks and operating the sponge was done manually, requiring 3 to 4 weeks. The training procedure was subsequently performed by a computer program, which also controlled the intensity of the clicks of 1 msec duration, presented continuously at a rate of 10/sec. The pulse generator was so designed that the output voltage was determined by the duration of pulses generated by the computer in discrete steps and applied to the generator within its linear range (10). Calibration by means of a microphone and oscilloscope showed that a single step very closely corresponded to a change in sound intensity of 1 db. The normal cat's threshold was found to be only a few db below that of humans.

This arrangement permitted on-line control of intensity according to a "titration" method (11). Following a correct response, the click intensity was reduced; a wrong response caused an increase in click intensity. It was soon observed, however, that after a series of fortuitous correct responses the sound intensity became so low that it remained below threshold for a substantial part of the session. This undesirable contingency was successfully eliminated by increasing sound intensity by two steps after a wrong response, an arrangement adopted for all subsequent tests.

Pharmacology: All of the psychopharmacological studies will be carried out in adult cats, both male and female. Cats will be injected 1 ml of one of the following: saline, 10-25 mg/kg piperidine HCl, 0.05-0.20 mg/kg nicotine sulfate; 0.010-0.050 mg/kg N-methyl 4-piperidylbenzilate. Throughout the testing period (at least 2 years), the cats will be maintained at their normal body weight, allowed access to water for only an hour daily, and given a vitamin supplement as needed. They will be tested 5 times/week.

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Reversal of nicotinic effects with the anticholinergic glycolate esters

One of the important problems in neuropharmacology is the extent to which nicotinic cholinergic systems are operative in the central nervous system. There are some neurons of the central nervous system, such as the Renshaw cells in the spinal cord (12), thalamus (13), corpus stratum (14), and medulla oblongata-pons (15), which appear to be nicotinic. Although there are some drugs, such as ganglionic blocking agents and certain cholinolytics which will antagonize some of the control effects of nicotine, there is a due need for more specific, potent agents.

As part of a program to investigate the structure-activity relationships of centrally active anticholinergic agents, literally hundreds of glycolate and related esters were synthesized by many collaborative investigators [see (1) and (2) for reviews]. We hope to test a few representative agents of this series for possible central 'antinicotinic' action. Included in this group will be a piperizanol, piperidinol, quinuclinol, and pyrollizadinol benzilate. All of these agents are presently in our possession. Some of these agents have been suspected of having central antinicotinic action (1).

Studies to date with nicotine

During the past few months the effect of nicotine on various psychophysical parameters has been investigated in 6 trained cats. The dose used was 0.1 mg/kg and the cats were tested in the computer-controlled behavioral paradigm as previously described.

The preliminary findings were as follows: 1) Some of the cats made an increased number of trials during the session, while the level of performance tended to be more constant. (Normally the number of trials tends to fluctuate somewhat from session to session.) 2) One of the interesting observations is the effect of nicotine on the relative time spent in the light and dark period during the trials. The dark period corresponds to the interval between a trial termination and onset, while the light interval corresponds to latency between stimulus onset and lever response. Dark period duration can then be used as index of response rate. The effects of nicotine can be seen in figure 2a and 2b, which is an oscillographic display of the relative time intervals automatically analyzed by the computer program. Without drug the dark period was greater than the mean light period; whereas, after a dose of 100 µg/kg nicotine the light period was actually greater than the dark period. The significance of this reversal in time intervals is not entirely known, and, to date, it has been observed in only one animal. It is probably indicative of an increased response rate as borne out by the fact that the number of trials/session was increased in this same cat by nicotine.

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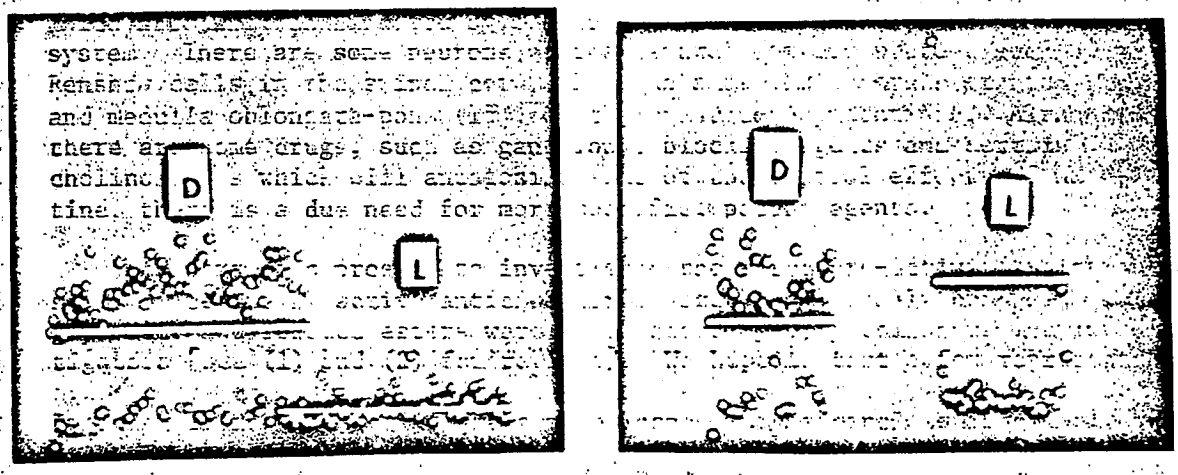
Reversal of nicotinic effects with the [illegible]



a b

Figures 2a and 2b.

In each photograph (oscillographic display) is represented the scatter of time points for an individual trial during dark (D) and light (L) period; the mean is represented by the line. Figure a is control and b is after 100 mg/kg nicotine.

3) The number of errors, i.e. the number of times the wrong lever was pressed, was unaffected by nicotine. 4) There was no overall effect on laterality, i.e. the preference of cats to press the left or right paw levers was not altered. Here again, with nicotine however, there was less variability in the performance from session to session. 5) There was no change in auditory threshold or in the relative time spent in the dark and light periods. 6) At the doses of nicotine employed there was no evident effect on the cats' appetite for food.

These preliminary findings suggest that at a dose of 0.1 mg/kg nicotine does not impair the cat's learned performance, but, in some instances, tends to make performance more constant. It will be interesting to determine how long-range chronic administration of nicotine influences any of the parameters, particularly with regard to their variability.

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References

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The physical facilities are located within the Center for Brain Research which occupies a total of 25,000 sq. ft. of space within the Medical Center. The space specifically allocated to this proposal consists of two behavioral experimental rooms comprising a total of 800 sq. ft. In one of these is housed the LINC-8 and ancillary equipment for on-line studies. A PDP-8 is also available along with the facilities of the Computer Center which are located in another part of the Medical Center. Within the Center for Brain Research are extensive neurophysiological, neurochemical, and neurohistological facilities as well as fully adequate animal quarters and an electronics shop.

11. Additional facilities required:

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

CURRICULUM VITAE

Leo G. Abood

Born:

REDACTED

Education: Ohio State University, B.S. Chemistry, 1943
University of Chicago, Ph.D. Pharmacology-Biochemistry, 1950

Experience:

Professor, Center for Brain Research, University of Rochester, 1965-
Professor, Department of Biochemistry, University of Rochester, 1965-
Professor of Neurophysiology and Biochemistry, U. of Illinois College of Medicine, 1963-65
Director of Research, Department of Psychiatry, U. of Illinois, College of Medicine, 1956-1965
Associate Professor of Neurophysiology and Biochemistry, University of Illinois, College of Medicine, 1954-63
Assistant Professor of Neurophysiology and Biochemistry, University of Illinois, College of Medicine, 1952-54
Instructor in Physiology, University of Chicago, 1950-52

Scientific Societies:

REDACTED

Major Research Interest:

Neurochemistry: Chemistry of Excitation; Neuropharmacology

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R: REDACTED MATERIAL

CURRICULUM VITAE

Karl Lowy

Born:

REDACTED

Education: University of Vienna Medical School, M.D., 1927

Experience:

Professor, Center for Brain Research, University of Rochester, 1960-
Professor, Department of Psychology, University of Rochester, 1968-
Clinical Associate Professor, Department of Otolaryngology, University of Rochester, 1959-
Senior Research Associate, Department of Psychology, University of Rochester, 1943-1968
Research Fellow, Department of Psychology, University of Rochester, 1941-1943
Internship, Medical Arts Center Hospital, New York City, 1938-1939
Instructor, Otolaryngology, University of Vienna, 1935-1938
Internship, Vienna, Allgemeines Krankenhaus, 1929-1930

Licensure, September, 1938, New York State

Scientific societies:

REDACTED

REDACTED

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13. Publications

Distribution of piperidine in the brain and its possible significance in behavior. L.G. Abood, Rinaldi and Eagleton, Nature, 191: 201, 1961.

Neuropharmacological investigations on the neural tissue-limb deplant of the salamander. Stuart R. Snider, Leo G. Abood and Ray S. Snider, Expt'l. Brain Res. 6: 81-88, 1968.

The role of proteins and lipids in membrane structure and funtion. L.G. Abood and A. Matsubara, in "Biogenic Amines and Physiological Membranes in Drug Therapy", J.H. Biel and L.G. Abood (eds.), Marcel Dekker, New York, 1971, pp. 1-33.

Interfacial adsorption of a psychotomimetic drug using liquid scintillation. H. Kimizuka and L.G. Abood, J. Pharmaceut. Sci., 62: 740-45, 1973.

Kinetics of Ca^{2+} adsorption and cationic selectivity with a synaptic membrane protein. L.G. Abood and W. Hoss, Biochimica et Biophysica Acta, 332: 85-96, 1974.

Evoked potential and microelectrical analysis of sensory activity within the cerebellum. Ray S. Snider and Karl Lowy, Fourth NASA Symposium on the role of vestibular organs in space exploration. October, 1968

Pulse generator with external amplitude control by computer. K. Lowy and L. Ota, Electroenceph. Clin. Neurophysiol., 25: 389-392, 1968.

Assessing the significance of averaged evoked potentials with an on-line computer: the split-sweep method. K. Lowy and B. Weiss, Electroenceph. Clin. Neurophysiol., 25: 177-180, 1968.

Influence of an anticholinergic psychotomimetic agent on behavior in cats controlled by an auditory stimulus. Karl Lowy, Bernard Weiss, and Leo G. Abood, Neuropharmacology, in press, 1974.

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
L. G. Abood K. Lowy	(summer, 3 months)	REDACTED
Technical Laboratory Assistant		REDACTED
	Sub-Total for A	R

B. Consumable supplies (by major categories)	
Animals and animal care	3,000
Chemicals, glassware	500
Sub-Total for B	3,500

C. Other expenses (itemize)	
Travel	300
Publication costs	200
Service contract on LINC-8	2,000
Sub-Total for C	2,500
Running Total of A + B + C	17,500

D. Permanent equipment (itemize)

Sub-Total for D	None
E	2,625
Total request	20,125

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppli.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	12,075	3,500	2,500	None	2,711	20,786
Year 3	12,679	3,500	2,500	None	2,802	21,481

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Mechanism of Action of Psychotomimetic Drugs	MH20142	$65,000	6/1/72 - 5/31/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Leo G. Abood

Signature Leo G. Abood Date 5/5/74

Telephone 716 (Area Code) 275-4024 (Number) (Extension)

Checks payable to

Roy B. Thompson, Senior V. Pres. and Treasurer

Mailing address for checks

University of Rochester

Rochester, New York 14627

Responsible officer of institution

Typed Name Richard E. Hufnail

Title Associate Director, Res. & Proj. Admin.

Signature Richard E. Hufnail Date 5/8/74

Telephone 716-275-4034 (Area Code) (Number) (Extension)

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#1006 - ALBANESE

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 5, 1974

Grant application No. 1006

PHARMACOLOGY

To The committee comprising Drs. Gardner, Jacobson, Sommers

Subject: Anthony A. Albanese, Ph.D., Burke Rehabilitation Center, White Plains, New York
New application No. 1006
"Effect of Cigarette Smoking on Utilization and Metabolism of Ascorbic Acid in Man"

History

CTR grants to this investigator, for more or less related studies, date back to 1962. There have been some gaps in support.

The enclosed competes as a new proposal.

Request

Application No. 1006 requests $24,250, for one year only.

Document submitted

Attached is application dated 7/25/74.

Comment

Dr. Hockett may wish to send you a written commentary.

F.W.N.

FWN:wg
Encl.

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A. INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
AUG 2 1974

Application for Research Grant
(Use extra pages as needed)

Date: 7/25/74

1. Principal Investigator (give title and degrees):

Anthony A. Albanese, Ph.D., Director of Nutrition and Metabolic Research Division

2. Institution and address:

Burke Rehabilitation Center
White Plains, New York 10605

3. Department where research will be done or collaboration provided:

Nutrition and Metabolic
Research Division

4. Short title of study:

"Effect of Cigarette Smoking on Utilization and Metabolism of Ascorbic Acid in Man".

5. Proposed starting date:

September 1, 1974

6. Estimated time to complete:

1 year

7. Brief description of specific research aims:

In 1961, Larson, Haag and Silvette raised questions concerning the effect of smoking on the availability of Vitamin C in man (1). Since then a number of reports have appeared which imply that there may be an untoward metabolic relationship in man between cigarette smoking and ascorbic acid levels in the urine, plasma and white blood cells (2). A careful review of this literature reveals that many of the confusing deductions from these studies arise primarily from choice of analytical methods and/or body fluids examined. Currently leucocyte determinations

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are widely regarded as the more reliable measure of vitamin C nutrition in man. However, the broad range of "normal values" reported by various investigators prompted us to examine the available methods for possible experimental errors. This search disclosed that the prevailing practice of reporting ascorbic acid content in terms of 10^8 white blood cells constitutes a major source of experimental error. To overcome this and other problems a new procedure has been developed and thoroughly tested for accuracy during the past 2 years. In our method ascorbic acid content is measured in terms of leucocyte protein, rather than a leucocyte count.

The main objective of the present proposal is to investigate the effects of cigarette smoking on vitamin C by our method rather than an arbitrary value of 10^8 cells. It is felt that this chemically definitive approach to the problem should resolve and clarify many of the existing ambiguities regarding the relationship between smoking and vitamin C metabolism.

Significance of Research Aims: In recent years the clinical interest in ascorbic acid nutrition has grown to public health proportions as a result of Linus Pauling's hypothesis that subnormal vitamin C nutrition may increase the incidence of upper respiratory infections (3). This concept which has been highly criticized is now finding significant support in the reports of Anderson and associates (4), Wilson and Loh (5) and more recently, by as yet, unpublished study of the USPHS.

Apart from these new clinical implications, ascorbic acid has long been known to be envolved in a number of physiologically important processes (6). Namely, it is a prerequisite for the formation of collagen, intercellular cement, dentine, cartilage, callus, osteoid tissue of the bone, blood vessel walls and the connective tissue; and is indispensable in the healing of wounds and the union of fractures. Ascorbic acid is important in tooth development and is a factor in resistance to infection. It is actively involved in certain vital functions of the body, such as carbohydrate and protein metabolism, cellular oxidation and hydrogen transfer. In its basic role, that of activator of tissue growth, its chief metabolic functions are thought to concern the adrenal cortex, pituitary gland, corpus luteum, brain, pancreas, liver and spleen, in which organs it is found in highest concentration. Ascorbic acid is also a strong reducing agent and an effective antioxidant.

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8. Brief statement of working hypothesis:

It has now been established beyond a reasonable doubt that qualitative or quantitative colorimetric test on urine, plasma or other body fluids are of limited, if not misleading, value for evaluation of vitamin C nutrition or detection of deficiencies in man (7,8). On the other hand, leucocyte vitamin C determinations are widely regarded as reliable and sensitive measures of ascorbic acid nutrition. However, in as yet unpublished studies we found that an accurate and clinically useful assessment of human vitamin C metabolism requires determination of both plasma and leucocyte protein ascorbic acid content. These investigations indicate plasma-leucocyte protein vitamin C ratios maybe the most significant metabolic parameter for evaluating the effects of endogenous and exogenous factors on the utilization and numerous biochemical functions of ascorbic acid. Accordingly, it is felt that a study of the effect of cigarette smoking on vitamin C in terms of changes of the plasma-leucocyte protein ascorbic acid ratios would provide data useful for clarification of existing controversies on this subject.

9. Details of experimental design and procedures (append extra pages as necessary)

Protocol: Measurements of ascorbic acid will be done on blood samples collected 2 hours after a sample breakfast of toast and coffee (200 calories) of 50 healthy normal male and female smokers of 1 pack or more of cigarettes and 50 non-smokers in the age range of 16 to 50 years. Details of duration and extent of smoking habits elicited by a questionaire will be correlated with the biochemical findings. The subjects selected for these studies will be paid volunteers who have signed an informed consent waiver. They will be hospital personnel who have passed the requirements of the annual employee health program.

Methods: Ascorbic acid levels in the leucocytes and plasma will be determined colorimetrically by the 2-4 dinitrophenyl hydrazine reaction. Ascorbic acid content of the leucocytes will be measured in terms of micrograms per 10^8 leucocyte as described by Denson and Bowers (9) and per milligrams of leucocyte protein by the method developed in our laboratory (10). It is expected that this dual analytical approach will serve to clarify such relationships as may exist between cigarette smoking and vitamin C nutrition in man.

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REFERENCES

1. Larson, P.S., Haag, H.B. and Silvette, H. Clin. Pharm. and Therapeutics 2, 80 1961

2. Pelletier, O. Nutrition Today 12-15 Autumn 1970

3. Pauling, L. Vitamin C and the Common Cold, San Francisco 1970; also Proc. Nat. Acad. Sci. 67, 1643 1970

4. Anderson, T.W., Reid, D.B.W. and Beaton, B.H. Canadian Med. Assoc. J. 107, 503 1972

5. Wilson, C.W. and Loh, H.S. Lancet, March 24, 1972

6. Goodhart, R.S. and Shils, M.E. Modern Nutrition in Health and Disease, Lea Febiger, Phil. 1973 pp. 244-255

7. Pelletier, O. Am. J. Clin. Nutr. 21, 1259 1968; 23, 520 1970

8. Calder, J.H., Curtis, R.C. and Fore, H. Lancet March 9, 1963 556

9. Denson, K.W. and Bowers, E.E. Clinical Science 21, 157 1961

10. Albanese, A.A. in press.

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10. Physical Facilities Available:

Nutrition and Metabolic Research Division previously visited and known to Dr. R. C. Hockett and Dr. McKean Cattell; and more recently Dr. Fredrick W. Nordsiek.

11. Additional Requirements:

None anticipated. Instrumentation required for the conduct of the described studies is on hand.

12. Biographical Sketches of All Principal and Professional Personnel:

See attached sheets.

13. PUBLICATIONS

Of a total of 229 publications, 77 have appeared in this decade and five invited scientific exhibits at national and international meetings.

Reviews and chapters in our area of competence have been prepared by invitation in the following text and reference books.

1. Albanese, A. A. General Nutrition of the Aging Patient in "Clinical Principles and Drugs in the Aging" (J. T. Freeman, editor), C. C. Thomas, Springfield, 1963, pp. 173-218.

2. Albanese, A. A. and Orto, L. A. The Proteins and Amino Acids in "Modern Nutrition in Health and Disease" (M. Wohl and R. Goodhart, editors), Lea and Febiger, Philadlephia, 3rd edition 1964; 4th edition 1968; 5th edition 1973

3. Albanese, A. A. Human Metabolism of Amino Acids and Fats in "The Art of Predictive Medicine" (W.L. Marxer and G. R. Cowgill, editors), C. C. Thomas, Springfield, 1967, pp. 99-104.

4. Albanese, A. A. Clinical Techniques for Evaluating Anabolic Agents in "Animal and Clinical Pharmacologic Techniques in Drug Evaluation" (P.E. Siegler and J.H. Mayer III, editors), Volume 2, Year Book Medical Publishers, Chicago, 1967, pp.763-778.

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5. Albanese, A. A. and Orto, L. A. Urinary Excretion of Amino Acids in "Newer Methods of Nutritional Biochemistry" (A. A. Albanese, editor) Volume III, Academic Press, New York, 1967, pp. 1-100.

6. Albanese, A. A. Nutritional and Metabolic Effects of Some Corticosteroids in Man in "Circadian Rhythms and New Aspects of Corticosteroids.

Research Reports 1970-1972

1. Albanese, A.A., Orto, L.A., Zavattaro, D.N., and De Carlo, R. Protein Metabolic Significance of Blood Ribonuclease Levels in Man. Nutr. Repts. International 4, 151 (1971).

2. Albanese, A. A., Orto, L.A., and Zavattaro, D.N. Nutritional and Metabolic Effects of Physical Exercise. Nutr. Repts. International 3, 165 (1971).

3. Albanese, A.A., Orto, L.A., Wein, E.H., and Zavattaro, D.N. Effect of Cigarette Smoking on Protein and Amino Acid Metabolism. I. Tryptophan. Nutr. Repts. International 5, 245 (1972).

4. Albanese, A.A., Lorenze, E.J., Orto, L.A., Wein, E.H., Zavattaro, D.N., and De Carlo, R. Nutritional and Metabolic Effects of Some Newer Steroids. VI. Serum Ribonuclease. N. Y. State J. Med. 72, 1595 (1972)

5. Albanese, A.A., Edelson, A.H., Woodhull, M.L., Lorenze, E.J., Wein, E.H. and Orto, L.A. Effect of Calcium Supplement on Serum Cholesterol, Calcium, Phosphorus and Bone Density of "Normal Healthy" Elderly Females. Nutr. Repts. International 8, 119 (1973)

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14. Budget:

A.	Salaries (Personnel by names)	% Time	Amount
	Professional		
	Anthony A. Albanese, Ph.D.	10%	2,400
	Technical		
	Evelyn H. Gruber, Chemical Technologist	100	13,500
	Secretarial services	20	2,000
		Sub-Total	$17,900
B.	Consumable Supplies (list by categories)		
	Reagents $600	$950	950
	Glassware 350		
		Sub-Total	$18,850
C.	Other Expenses (itemize)		
	Publication costs $600	$2,200	
	Travel to meetings 600		
	Fees for test subjects 1,000		
		Sub-Total	$21,050
D.	Permanent Equipment (itemize)		
	None		$21,050
E.	Indirect costs (15% of A+B+C)	$3,200	3,200
		TOTAL	$24,250

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Ascorbic Acid Needs of the Elderly	Private Foundation	$8,960	July 1, 1974 June 30, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None	None		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Anthony A. Albanese, Ph.D.

Signature Anthony A. Albanese Date 7/25/74

Telephone 914-948-3676
Area Code Number Extension

Checks payable to

Burke Rehabilitation Hospital

Mailing address for checks

White Plains New York

Responsible officer of institution

Typed Name Fletcher H McDowell

Title Medical Director

Signature Fletcher H McDowell Date 7/25/74

Telephone 914 948-1765
Area Code Number Extension

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#623C - ESSMAN

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 12, 1974

Grant application No. 623C

PHARMACOLOGY

To: The committee comprising Drs. Gardner, Liebow, Sommers

Subject: Walter B. Essman, M.D., Ph.D., Queens College of the City University of N. Y.
Continuation application No. 623C
"Studies of Nicotine Action upon Memory Consolidation"

History

CTR grants for this study began in 1968; with renewals and continuations support has been continuous.

The current level of support is approximately $22,000 a year.

Request

Application No. 623C requests $23,196. The face sheet indicates that three years are estimated as the time of completion.

As this is a "continuation" application, it competes on the same basis as a new proposal.

Documents Submitted (attached)

1. Application dated 8/5/74.
2. Progress Report January 16, 1973 to August 1, 1974.

Comment

Dr. Essman keeps CTR well supplied with reprints of his numerous publications; any of these will be promptly forwarded upon request.

F.W.N.

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Encl.

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THE COUNCIL FOR TOBACCO RESEARCH -- U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
AUG 12 1974

APPLICATION FOR RESEARCH GRANT
(Use extra pages as needed) DATE: 8/5/74

1. Principal Investigator (give title and degrees):

Walter B. Essman, M.D., Ph.D.
Professor of Psychology & Biochemistry

2. Institution & address: Queens College of the City University of N.Y. 65-30 Kissena Blvd. Flushing, New York 11367 and The Research Foundation of the City University of N.Y. 1411 Broadway New York, New York 10018

3. Department(s) where research will be done or collaboration provided:

Psychology, Biochemistry

4. Short title of study: "Studies of Nicotine Action Upon Memory Consolidation"

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: 36 months (3 years)

7. Brief description of specific research aims:

The present protocol represents an extension of research investigations of regional, cellular and subcellular central nervous system sites altered by nicotine and/or its metabolites and the process of memory consolidation (i.e. neural events by which the memory trace becomes fixated and stored within the central nervous system). Specific studies will further explore the hypothesis that nicotine and several of its biologically active metabolites may serve to block the molecular effects of 5-hydroxytryptamine at selected receptor sites. The studies will further deal with mechanisms by which nicotine and its metabolites prevent 5-HT induced inhibition of protein synthesis which has been shown to serve as an amnesic event. Specifically, this hypothesis will be investigated on the basis of:

1) developmental effects of nicotine and metabolites;

2) strain differences in nicotine and metabolite effects; 1003544434

3) strain and age interactions and memory consolidation;

4) and, the inter-relationship of age and strain variables in the process of memory consolidation effects of nicotine and metabolite effects on a cellular level.

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7. continued:

Because the neuropharmacology of nicotine appears significant in that it offers import insight into basic mechanisms of memory consolidation, the present project also holds further significance in the clarification of those molecular mechanisms identified with disruption of the memory consolidation process, i.e., retrograde amnesia.

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8. Brief statement of working hypothesis:

Nicotine and several of its biologically active metabolites capable of exerting central nervous system effects are capable of interfering with receptor effects of brain 5-hydroxytryptamine which, in appropriate circumstances, inhibit synaptic protein synthesis and interfere with the memory consolidation process. Thereby, such compounds can alter the time course over which memory consolidation occurs and facilitate the process. Such facilitation of memory consolidation by nicotine and metabolites will be regulated by the age of the animal within specific strains; i.e., certain strains will be more or less susceptible to the facilitative effects of these drugs. Similarly, the interactions between nicotine and metabolites of 5-HT induced inhibition of synaptic protein synthesis will be age and strain dependent.

9. Details of experimental design and procedures (append extra pages as necessary)

Mice of all three strains to be utilized in these studies will be conditioned to establish a stable passive avoidance response conditioned in a single training trial, utilizing a method (Essman and Alpern, 1964) established as effective for this purpose. The amnesic effects of a variety of agents and/or events presented in close temporal proximity with the acquired experience in this situation results in a reduced incidence of retention measured 24 hours or more later. Amnesia in this situation is defined by inability of the animal to perform the avoidance response to which it was previously trained.

Mice will be utilized from stock either obtained commercially or specially bred in institution vivarium facilities in order to maintain persistent controls over age differences. The strains to be utilized in the present study will be CF-1S (Carworth Farms), which have been previously utilized in past studies; C57/BL6J, a mouse strain previously shown to exhibit superior conditioning ability and long term retention of conditioned behavior, in addition to showing a decreased

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susceptibility to the amnesic effects of several training treatments which usually result in a retrograde amnesia. The third strain of mouse to be utilized in the series of experiments will be DBA-2 strain, which has proven to be a poorly conditioned animal that shows a markedly increased susceptibility to the amnesic effects of several agents or events.

Animals from the designated strains will be utilized at specific ages ranging from 16 to 35 days and these will deal with (1) The effects of nicotine and active metabolites such as cotinine and 3-pyridylacetic acid upon the retrograde amnesic effects respectively induced by: (a) transcorneal ECS (see Essman, 1973, #5, Pg. 3b; (b) intrahippocampal 5-hydroxytryptamine (Essman, 1973, #9, Pg. 3b2, and (c) intraperitoneal injection of short-chain fatty acids. All three of the above treatments have been shown to exert a time dependent retrograde amnesia in CF-1S strain mice and also to elevate brain 5-hydroxytryptamine content and cause an inhibition of brain protein synthesis. Extensive publications have supported these phenomenon in the case of the first two treatments and the third treatment holds particular significance in that increased short-chain fatty acid concentration has been implicated in several clinical entities, such as Wilson's dieease, hepatic coma, hepatic cirrhosis and metabolic convulsions -- all of which involve altered sensorial and memory function. Intraperitoneal injection of 15 mg/kg of octanoic acid induces coma rapidly (70.3 ± 4.6 seconds with an average duration of 17.7 ± 3.7 minutes); such short-chain fatty acid induced comas produce a retrograde amnesia, elevation of brain 5-hydroxytryptamine and inhibition of protein synthesis which is antagonized by both nicotine as well as its major metabolite, cotinine.

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In the proposed experiments, mice from three designated strains will be treated either immediately, 60 seconds, 10 minutes, or 60 minutes following conditioning as previously described, and a temporal gradient for the retrograde

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amnesic effects of such treatments will be generated as a function of age.

Based upon such baseline data, the dosage and drug treatment interval for nicotine, cotinine, 3-pyridylacetic acid, will be assessed in experiments that will allow for (2) Specification of the temporal relationship between nicotine and/or metabolic effects upon cerebral amine metabolism and amine metabolite inhibition of protein synthesis.

Studies will specifically test the hypothesis that strain and age-dependent antagonism of retrograde amnesia by nicotine and several of its active metabolites depends upon the ability of this drug to block metabolic events associated with the amnesic stimulus. Such events specifically included the relevence and increased site accumulation of 5-HT at the presynaptic nerve ending and mediation, thereby of unhibition of protein synthesis in these regions. For the three amnesic agents utilized, the age and strain difference will be considered with respect to (a) differences in 5-HT metabolism in the synaptic area; (b) alterations in protein synthesis in these regions measured by the incorporation of C^{14}-leucine into proteins and (c) the time relationship between these two events. Under these conditions, effects of either nicotine, cotinine, and 3-pyridylacetic acid will be determined with the former compound administered in doses up to 1 mg/kg body weight and the latter two compounds administered up to doses of 0.3 mg/kg.

A final series of studies will concern (3) The cellular effects of nicotine and active metabolites during late development. It has been shown that aging, particularly in rodents, contributes appreciably to the changes in retentive ability for retentive behavior and to the degree to which the potency of certain amnesic stimuli may be manifest. It is the purpose of these series of experiments to inter-relate the effects of nicotine, cotinine and 3-pyridylacetic acid, based

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9. continued:

upon dose response elaboration, to the regional changes in 5-HT metabolism and synaptic protein synthesis in old mice. Mice from the three designated strains will be maintained in laboratory colonies until the age of 120 days, when behavioral testing will establish: (a) changes in their conditioning for passive avoidance behavior, (b) changes in their susceptibility to the three amnesic events to be utilized, and (c) changes in endogenous 5-HT and protein synthesis. It is hypothesized that aging will contribute to reduced resistance to stimulus-induced amensia, measured both behaviorally with the conditioning technique described and biochemically by the changes in 5-HT metabolism and synaptosomal protein synthesis. Under these conditions, it is hypothesized that the dose, duration of effect and potency of nicotine and active metabolites will be altered so that such effects may be more apparent in aged mice.

These relationships will be studied in several experiments that allow for a detailed statement of the relationship between both early development as well as aging as contributants to the nicotine mediated changes in memory consolidation and the biochemical events requisite for that process.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Two air-conditioned laboratories with adjoining vivarium facilities are currently in use by the investigator. All equipment necessary for the behavioral and biochemical procedures involved with this proposal are available and no additional equipment needs are anticipated for the duration of this project.

11. Additional facilities required:

NONE

12. Biographical sketches of investigator(s) and other professional personned (append):

See Page 3a.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available.

See Page 3b.

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WALTER B. ESSMAN

Education:

Ph.D. - University of North Dakota, 1957 (Major: Psychology; Minor: Medical Sciences)

M.D. - (Cum Laude) - University of Milan, 1972 - when did he take time out to earn this?

M.A. - University of North Dakota, 1955

B.A. - New York University, 1954

Experience:

Professor (Psychology, Biochemistry), Queens College of the City University of New York, 1967 - Present.

Associate Professor, Queens College of the City University of New York, 1965-1966.

Assistant Professor, Queens College of the City University of New York, 1962-64.

Research Professor, Neurology, Mt. Sinai School of Medicine, New York, 1972 - Present.

Research Associate, Laboratory of Neurochemistry, Mt. Sinai School of Medicine, New York, 1966 - 72.

Research Fellow, Laboratory of Neurochemistry, Mt. Sinai Hospital, New York, 1964 - 66.

Research Assistant Professor, Dept. of Rehabilitive Medicine, Albert Einstein College of Medicine, 1962-63.

Research Associate, Dept. of Physiology, Albert Einstein College of Medicine, 1961-63.

Senior Post-Doctoral Fellow, Neurophysiology, Albert Einstein College of Medicine, 1959-61.

Director, Psychophysiological Research, U.S. Army Surgical Research Unit, 1958-59.

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13. Publications (5 most recent):

1. Essman, W.B. Brain 5-hydroxytryptamine and memory consolidation. In: Gessa, G. L., and Sandler, M. Advances in Biochemical Psychopharmacology, Volume 11, Serotonin: New Vistas. Biochemistry and Behavioral and Clinical Studies. Raven Press, 1974, Pgs. 265-274.

2. Essman, W.B. Amine mediated retrograde amnesia: factors effecting cerebral protein synthesis. In: Bogoch, S. (Ed). Biological Diagnosis of Brain Disorders. N.Y.: Spectrum Publications, 1974.

3. Essman, W.B. Effects of ECS on cerebral protein synthesis. In: Fink, M., Kety, S.S., McGaugh, J., & Williams, T. (Eds.). The Psychobiology of Convulsive Therapy. Washington, D.C.; V.H. Winston & Sons, 1974, Pp. 237-249.

4. Essman, W.B. Pharmacological alterations of the retrograde amnesic effect of electroconvulsive shock: the modification of forebrain 5-hydroxytryptamine in mice. Pharm. Res. Commun., 1973, 5: 295-302.

5. Essman, W.B. Neurochemistry of Cerebral Electroshock. New York: Spectrum Publications, 1973.

Relevant Publications: 1971-1973

1. Essman, W.B. and Nakajima, S. Experimentally-induced retrograde amnesia: some neurochemical correlates. In: Essman, W.B. & Nakajima, S. (Eds.). Current Biochemical Approaches to Learning and Memory. N.Y.: Spectrum Publications, 1973, Pp. 159-188.

2. Essman, W.B. Effetti dell'elettroshock sulla neurochimica del sistema nervaso centrale, 1. Rass. Clin. Scient., 1972, 48: 361-370.

3. Essman, W.B. Biphasic effects of nicotine on ECS-induced retrograde amnesia in mice. Psychol. Rep., 1973, 32: 658. (with S.G. Essman).

4. Essman, W.B. Retrograde anmesia and cerebral protein synthesis: initiation and inhibition by 5-Hydroxytryptamine. Totus Homo, 1972, 4: 61-67.

5. Essman, W.B. Age-dependent effects of 5-hydroxytryptamine upon memory consolidation and protein synthesis. Pharmacol. Biochem. & Behavior, 1973, 1: 7-14.

6. Essman, W.B., Whittaker, V.P. and Dowe, G.H.C. The isolation of pure cholinergic synaptic vesicles from the electric organs of elasmobranch fish of the family Torpedinidae. Biochem. J., 1972, 128: 833-846.

7. Essman, W.B. Discussion of neurochemistro of benzodiazepines. In: Garattini, S., Mussini, E., & Randall, L.O. (Eds.). The Benzodiapzpines. N.Y.: Raven Press, 1973, Pp. 281-283.

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8. Essman, W.B. Nicotine-related neurochemical changes: some implications for motivational mechanisms and differences. In: Dunn, W.J., Jr., (Ed.). Smoking Behavior, 1973, Pp. 51-65.

9. Essman, W.B. Neurochemical modulation of experimentally induced retrograde amnesia. Confinia Neurol., 1973, 35: 1-22.

10. Essman, W.B. Changes in the amnesic and aversive properties of avoidance conditions with chlordiazepoxide. Pharmacol. Biochem. & Behavior, 1973, 1: 125-128.

11. Essman, W.B. Subcellular actions of benzodiazepines. In: Garattini, S., Mussini, E. & Randall, L.O. (Eds.). The Benzodiazepines. N.Y.: Raven Press, 1973, Pp. 177-190.

12. Essman, W.B. Neurochemical changes in ECS and ECT. Seminars in Psychiatry, 1972, 4: 67-79.

13. Essman, W.B. and Heldman, E. Impairment of avoidance acquisition and altered regional brain amine levels in mice with eremic endotoxemia. Physiol. Behav., 1972, 8: 143-146.

14. Essman, W.B. Changes in cholinergic activity and avoidance behavior by nicotine in differentially housed mice. Int. J. Neurosci., 1971, 2: 199-206.

15. Essman, W.B. and Essman, S.G. Cholinergic mechanisms and avoidance behavior acquisition: effects of nicotine in mice. Psychol. Rep., 1971, 29: 987-993.

16. Essman, W.B., Essman, S.G. and Golod, M.I. Metabolic contributions to gastric ulcerogenesis in mice. Physiol. Behav., 1971, 7: 509-516.

17. Essman, W.B., Barker, L.A., Dowdall, M. & Whittaker, V.P. The compartmentation of acetylcholine in cholinergic nerve terminals. In: Heilbonn, E., and Winter, A. (Eds.). Drugs and Cholinergic Mechanisms in the C.N.S. Stockholm: Research Institute of National Defense, 1971.

18. Essman, W.B. The role of biogenic amines in memory consolidation. In: Adam, G. (Ed.). The Biology of Memory. N.Y.: Plenum Press, 1971, Pp. 213-238.

19. Essman, W.B. Isolation-induced behavioral modification: some neurochemical corellates. In: Sturman, M.G., McGinty, D.J. and Adinolfi, A.M. (Eds.). Brain Development and Behavior. N.Y.: Academic Press, 1971, Pp. 265-276.

20. Essman, W.B. Drug effects and learning and memory processes. In: Garattini, S. and Shore, P. (Eds.). Advances in Pharmacology and Chemotherapy. N.Y.: Academic Press, 1971, Pp. 241-330.

21. Essman, W.B. Behavioral correlates of acute renal failure. In: Coletti, R.B. and Krueger, K.K. (Eds.). Proc. of Workshop on Behavioral Bioassay in Uremia. Washington, D.C., U.S. Gov't Printing Office, 1971, Pp. 18-30.

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22. Essman, W.B. Drugs affecting facilitation of learning and memory. In: Rubin, A.L. (Ed.). Search for New Drugs. N.Y.: Dekker, 1972, 385-406.

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24. Essman, W.B. Memory and amnesia changes induced by nicotine and its metabolites. In: Votava, Z. (Ed). Neuropsychopharmacology, 1971.

25. Essman, W.B. Metabolic and behavioral consequences of nicotine. In: Smith, W.L. (Ed.). Cerebral Function Development and Drug Action. Springfield: Chas. Thomas, 1972, Pp. 273-287.

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R: REDACTED MATERIAL

14. First year budget:

	% Time	Amount
A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)		
Dr. Walter B. Essman		REDACTED
Technical		
Traum, John - Technical Assistant		REDACTED
Rosenthal, Richard -- Assistant		
+ 20% Fringe Benefits		REDACTED
Sub total for A		
B. Consumable supplies (by major categories)		
Animal purchases		$ 800.
" cleaning, bedding		600.
" chemicals, glassware		840.
disposable cages		450.
Sub total for B		$ 2,690.
C. Other expenses (itemize)		
6th Annual Winter Conference for Brain Research, January, 1975, Vail, Colorado		
Endocrine Society, Chicago, Illinois, June, '75		
American College of Neuropsychology, San Juan, Puerto Rico, December '75		
Sub total for C		$ 1,100.
Running Total of A+B+C		$20,170.
D. Permanent equipment (itemize)		
NONE		
Sub total for D		----
E. Indirect costs (15% of A+B+C)	E	$ 3,026.
Total Request		$23,196.

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Metabolic Response to Stress -- Tobacco Smoke Interactions	RF-09165 C.T.R. #836A	$40,000.	10/1/73 - 9/30/74
"Role of Nicotine Action Upon Memory Consolidation	RF-09104 C.T.R. #632BR1 (Continuation of carry over funds)	$14,911.79	2/1/74 - 12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to:

Research Foundation of C.U.N.Y.

Mailing Address for checks:
Mr. Paul Segall, Comptroller
Research Foundation of C.U.N.Y.
1411 Broadway
New York, New York 10018

Principal Investigator

Typed Name: Walter B. Essman, M.D., Ph.D.

Signature: Walter B. Essman Aug 5, 1974

Telephone: (212) 544-6421

Responsible Officer of Institution

Typed Name: 1. Hannah Petzenbaum
2. David H. Speidel

Title: 1. Research Fndtn.- Q.C.
2. Asst. Dean of Faculty Q.C.

Signature: 1. [illegible] Date: 8/5/74
2. [illegible]

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The Council for Tobacco Research - U.S.A. Grant, "Studies of Nicotine Action Upon Memory Consolidation."

Walter B. Essman, M.D., Ph.D.
Principal Investigator
Queens College of the City University
of New York
65-30 Kissena Blvd.
Flushing, New York 11367

PROGRESS REPORT

January 16, 1973 to
August 1, 1974.

STUDIES OF NICOTINE ACTION UPON MEMORY CONSOLDIATION

Within the past 17 months, or since the submission of a previous progress report for this research project (January 16, 1973 - Aug. 1, 1974), the research has been specifically concerned with the interaction of putative neurotransmitters and the central effects of nicotine and several of its biologically active metabolites. Changes in brain serotonin (5-hydroxytryptamine) metabolism have, in previous research, been established in conjunction with several amnesic agents and/or events, several of which appear to be temporally contiguous with the amnesic associated inhibition of cerebral protein synthesis. Such effects, particularly as brought about experimentally through the administration of electroconvulsive shock (ECS) have been antagonized or blocked through the administration, prior to administration of the amnesia stimulus of nicotine. These effects have been documented in previous publications from our laboratory. We have also demonstrated that several metabolites of nicotine, namely cotinine and 3-pyridylacetic acid, are capable of antagonizing the amnesic effects brought about by post-training ECS and similarly alter the effects of such ECS upon brain 5-HT metabolites. These effects have also been shown to be time-dependent to the extent that 45 minutes appears to be optimal following nicotine injection, after which the amnesic properties of ECS are antagonized, whereas 15 minutes after treatment, the effects appear to be potentiated. In Figure 1, these behavioral data have been summarized for nicotine and several of its derivatives.

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As may be observed in Figure 2, the ratio of 5-HT and its metabolites in brain, 5-hydroxyindoleacetic acid (5-HIAA), is regionally modified following nicotine treatment and this effect is certainly consistent with observations that have been summarized in Figure 3. They show that nicotine administration procedures reduced turnover rate and increased turnover time for this brain amine.

The regional concentration of 5-HT and its metabolite in several regions of the mouse brain at those times at which different potential or antagonistic effects of nicotine have been shown in the amnesic paradigm, summarized in Table 1, from which it may be noted that the time difference accounts to a considerable extent for the change in regional 5-HT metabolism.

The question of the cellular specificity of this change has also been explored for different regions of the mouse brain that have been fractionated to yield cell pools enriched with their neuronal cell bodies or glia. These data, summarized in Figure 4 indicate that the neuronal as well as glial pools of the amine have been increased as a consequence of nicotine treatment. The possible bearing of this finding to the regional effects of nicotine and several related compounds to its interaction with such amnesic stimuli as ECS may be observed in Table 2, as compared with saline control treatment, significant changes in several brain regions apparently participated in the mediation of nicotine effects, have been documented.

The neurochemical consequences of cerebral electroshock have been explored and analyzed in a recent monograph wherein the interaction with nicotine, which may be relative to memory consolidation phenomenon, have been explored.

The age-dependent nature of amnesic effects produced by several experimental conditions and their relationship to the process of cerebral protein synthesis

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and the effects thereupon of nicotine, have also been considered. From these experiments, the concept of a "Critical Age" phenomena has emerged, such that in the 17-day-old CF-1S strain mouse, an alalog of the central effects of nicotine observed in older mice, behaviorally as well as biochemically, has emerged. It was shown in this context that the intracranial administration of 5-hydroxytryptamine (5-HT) was capable of inducing an age-dependent amnesic effect, as well as an age-dependent effect upon the synthesis of cerebral protein.

The cholinergic effects of nicotine, although well-known, were further investigated on a subcellular level to specifically examine changes in the size of various cholinergic pools in the cerebral cortex. These results have indicated that administration of nicotine leads to a decrease in the acetylcholine content of both bound and vesicular storage pools without any alteration in the concentration of free acetylcholine.

The effects of nicotine were also examined in relation to the differential effect of this compound among environmentally conferred differences in learning ability. These previously published data have indicated that task-specific facilitative effects of nicotine exert different effects both behaviorally and metabolically depending upon the endogenous behavioral and metabolic baseline upon which they are superimposed. Such a model has been derived utilizing differentially housed mice. In animals treated with radioactive nicotine, it was found that isolated animals showed a greater brain uptake, for several regions of nicotine, as compared to group housed mice, where the only appreciable uptake occurred in the basal ganglia; the latter change, however, was markedly below the uptake of nicotine shown by isolated animals. Nicotine metabolites, specifically cotinine, were also found to be present in considerably greater quantities in several regions of the brain of isolated animals. This finding would be consistent with our previous suggestion that the facilitative effect of nicotine treatment upon the learning

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ability of behaviorally retarded mice (as result from early isolation housing) may be dependent upon a biologically active metabolite of nicotine, rather than to nicotine per se. A likely candidate for such a metabolite is cotinine, which we have observed to exert potent biochemical and behavioral effects in earlier studies.

In several studies we have also examined the regional differences in cerebral protein synthesis exerted by nicotine at several subcellular sites. In this regard, we have conclusively established that microsomal protein synthesis in mouse cerebral cortex is significantly augmented as a consequence of nicotine treatment, and further that the protein synthesis inhibition which may be environmentally produced by the stress of isolation housing may be completely reversed at the microsomal level by treatment with nicotine. In this respect, preliminary studies of mitochondrial proteins from mouse cerebral cortex have indicated temporal qualitative and quantitative differences in the effects of nicotine. It is our purpose to further examine these changes and characterize them with respect to some of the metabolites of this compound.

In a final series of studies, progress of which may be indicated at this point, it was shown that an experimentally-induced retrograde amnesia effected in mice with electroconvulsive shock could be antagonized or potentiated depending upon the interval of time between nicotine treatment and the post-conditioning amnesic event. In this regard, it was shown that conditioned response retention was reduced below control levels if nicotine treatment was given 15 minutes prior to ECS, whereas if such treatment was effected 45 minutes prior to treatment, approximately 80% of the animals showed antagonism toward the amnesic effect. These results again are consistent with our earlier findings and provide further support for the general working hypothesis that the role of nicotine in memory

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consolidation is to provide temproally related facilitative effects as are dependent upon aminergic and protein synthetic interactions in the central nervous system.

Currently under development are a series of procedures designed to provide for *in vitro* assessment of *in vivo* effects of nicotine and amnesic changes in relationship to the distribution of cerebral amines and the synthesis of cerebral proteins. These studies have focused upon microsomal systems, isolated from several regions of the mouse brain and have indicated that several of the parameters by which nicotine metabolism, central distribution of nicotine and/or metabolites and behavioral effects of nicotine and/or metabolite specific memory consolidation phenomenon have been regulated, such as differential housing, provide for differences observed utilizing *in vitro* protein synthesis methods. More specifically in this context, it has been observed that *in vivo* depletion of 5-hydroxytryptamine (5-HT), differential housing, or *in vivo* treatment, acutely or chronically with nicotine, can significantly alter the rate of microsomal protein synthesis measured *in vitro*, or modify the inhibitory effect of 5-HT upon such protein synthesis.

The significance of memory consolidation and the model proposed for one facet of its operation relates to other conditions to which memory-like mechanisms may, by analogy, by cited. For example, the very interesting work of D. Clouet has indicated that rats that developed an addictive state in narcotic drugs and are subject to withdrawl symptoms subsequent to drug abstinence do both things in this spectrum if treated with anti-biotics such as puromyacin. If such a regimen is carried out, addiction does not develop, dose habituation does not occur, and there is an absence of withdrawl signs. This may perhaps illustrate one form of memory in the sense that a state-dependent physiology phenomenon

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may be obviated for the synthesis of proteins with which it must be apparently induced has been inhibited. Another example may be found in the area of state-dependent learning, wherein animals treated with barbiturates such as pentobarbitol or phenobarbitol are capable of learning responses while under the influence of such barbiturates; further, such responses are manifest when tested in the non-barbiturate state. When barbiturates are again administered to such animals, such may be elicited in the barbiturate state. A parallel between these latter experiments and animal tissue inhibition in the study in progress is that such barbiturates do elevate brain 5-HT content and 5-HT level drops following barbiturate withdrawal. This, based upon our previous data, shows a relationship between 5-HT level and protein synthesis; the analogy may be extended further to suggest that inhibition of protein synthesis is 5-HT dependent and thereby state dependency is a related event to protein synthesis dependency; both are 5-HT metabolites.

With regard to our more recent findings concerning short chain fatty acids, it might be pointed out that the particular relevance of these substances to the present project and to the hypothesis to be tested in the proposed research is that these compounds are produced in excess with a variety of hepatic dysfunctional states, such as cirrhosis, hepatolenticular degeneration, coma, and drug induced toxicity. Experimental mediation of coma by administration of short chain fatty acids has been shown with such agents as butyric acid, valeric acid, and octanoic acid in doses ranging from 10μM per kg to 25μM per kg. It was further apparent that doses capable of rapidly inducing coma, of brief duration, were also capable of mediating a retrograde amnesia for a passive avoidance conditioned response increasing the concentration of brain 5-HT leading to a marked reduction in the synthesis of proteins in several regions of the mouse brain. These studies have further provided a base line upon which subsequent investigations are contemplated.

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PUBLICATIONS RESULTING FROM THIS PROJECT

1. Essman, W.B. Contributions of differential housing to brain development: some implications for sleep behavior. In: Clemente, C.D., Purpura, D.P. and Mayer, F.E. (Eds.). Sleep and the Maturing Nervous System. New York: Academic Press, 1971, Pp. 99-107.

2. Essman, W.B. Drugs affecting facilitation of learning and memory. In: Rubin, A.L. (Ed.). Search for New Drugs. New York: Dekker, 1972, 385-406.

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14. Essman, W.B. Effetti dell'elettroshock sulla neurochimica del sistema nervaso centrale, II. Rass. Clin. Scient., 1973, 49: 5-23.

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16. Essman, W.B. Pharmacological alterations of the retrograde amnesic effect of electroconvulsive shock: the modification of forebrain 5-hydroxytryptamine in mice. Pharm. Res. Commun., 1973, 5: 295-302.

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19. Essman, W.B. Effects of ECS on cerebral protein synthesis. In: Fink, M., Kety, S.S., McGaugh, J. & Williams, T. (Eds.). The Psychobiology of Convulsive Therapy. Washington, D.C., V.H. Winston & Sons, 1974, Pp. 237-249.

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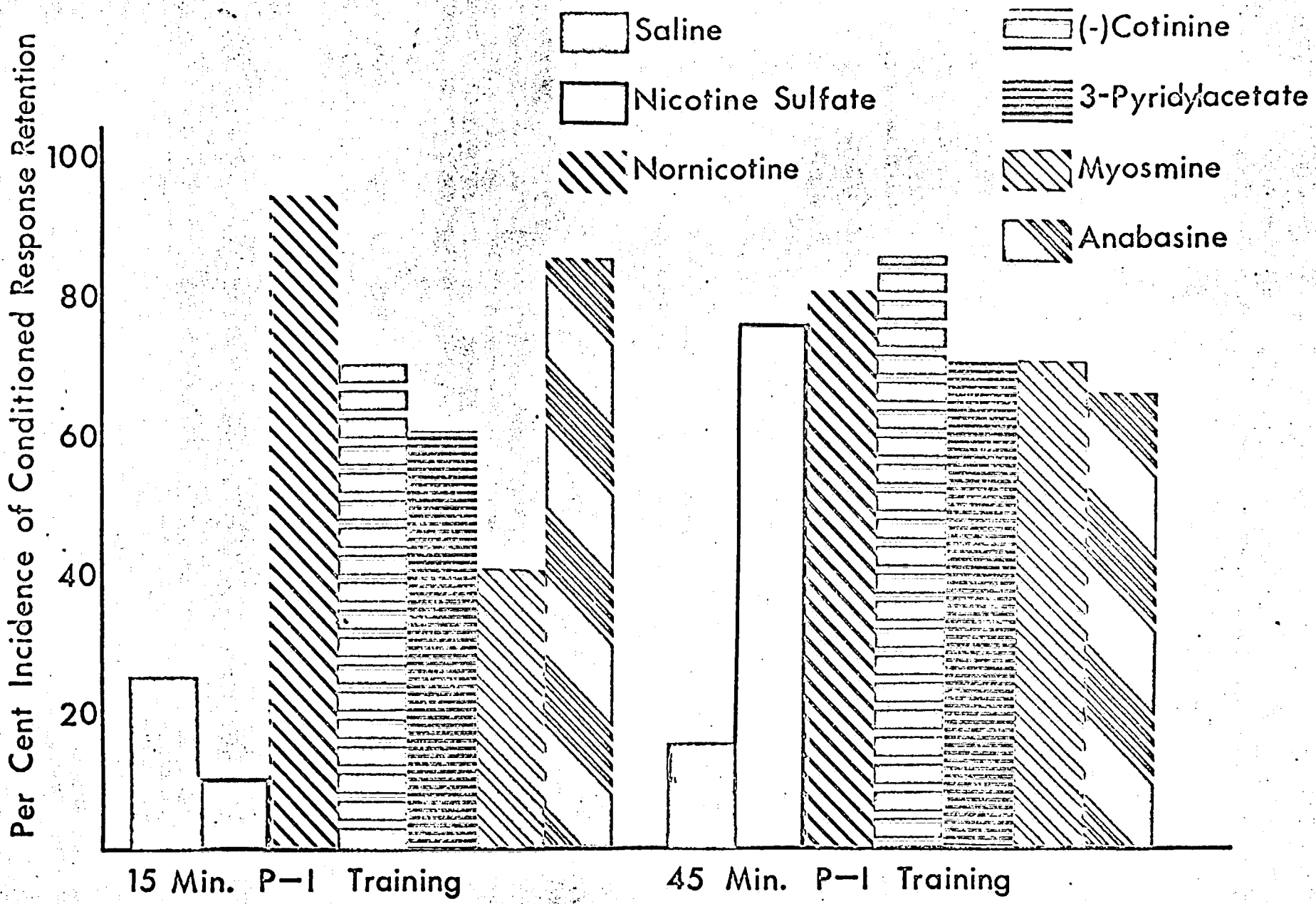

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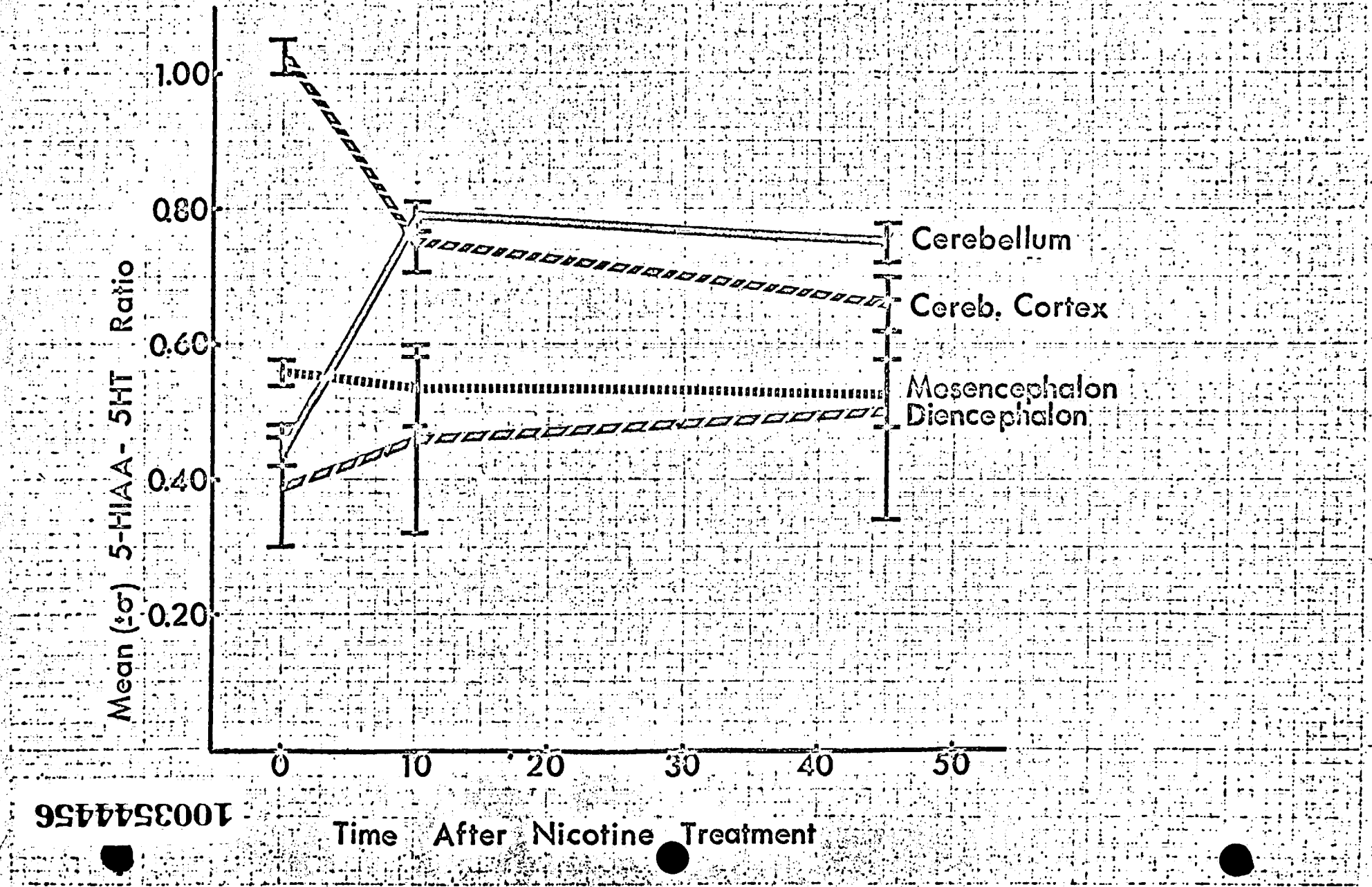

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Mean(±σ) Regional 5-HT Concentration (μg/g)

0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80

Saline 0.9%

Cerebral Cortex

Corpus Callosum

Diecephalon

Limbic System

Cerebellar Cortex

Nicotine Sulfate (1.0 mg/kg)

Cerebral Cortex

Corpus Callosum

Diencephalon

Limbic System

Cerebellar Cortex

Neuronally-Enriched

Glia-Enriched

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TABLE 1.

MEAN ($\pm\sigma$) REGIONAL CONCENTRATION OF 5-HYDROXYTRYPTAMINE (5-HT) AND 5-HYDROXYINDOLEACETIC ACID (5-HIAA) FOLLOWING SALINE-OR DRUG-TREATMENT*

BRAIN AREA	TIME POST-TREATMENT					
	15			45		
	SALINE	NICOTINE	COTININE	SALINE	NICOTINE	COTININE
CEREBRAL CORTEX: 5-HT	0.38 (0.06)	0.42 (0.01)	0.33 (0.04)	0.39 (0.04)	0.66 (0.04)	0.28 (0.03)
5-HIAA	0.52 (0.19)	0.55 (0.06)	0.43 (0.05)	0.12 (0.02)	0.37 (0.02)	0.36 (0.04)
MESENCEPHALON: 5-HT	0.33 (0.04	0.82 (0.02)	0.83 (0.05)	0.33 (0.04)	0.64 (0.06)	0.33 (0.05)
5-HIAA	0.21 (0.02)	0.45 (0.02)	0.36 (0.04)	0.18 (0.03)	0.35 (0.06)	1.00 (0.12)
DIENCEPHALON: 5-HT	0.33 (0.04)	0.96 (0.20)	0.56 (0.04)	0.33 (0.05)	0.90 (0.15)	0.28 (0.05)
5-HIAA	0.15 (0.03)	0.45 (0.09)	0.29 (0.04)	0.14 (0.03)	0.45 (0.18)	1.07 (0.11)

* Concentrations expressed as $\mu g/g$.

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MEAN ($\pm\sigma$) REGIONAL CONCENTRATION OF BRAIN 5-HYDROXYTRYPTAMINE (5-HT) AND 5-HYDROXYINDOLEACETIC ACID (5-HIAA) IN DRUG-TREATED MICE GIVEN ELECTROCONVULSIVE SHOCK (ECS) OR SHAM-ELECTROCONVULSIVE SHOCK ($\overline{ECS}$).

TREATMENT AND REGION SAMPLED	POST-INJECTION (P.I.) CONDITION							
	ECS 15' P.I.		ECS 45' P.I.		$\overline{ECS}$ 15' P.I.		$\overline{ECS}$ 45' P.I.	
	5-HT	5-HIAA	5-HT	5-HIAA	5-HT	5-HIAA	5-HT	5-HIAA
NORNICOTINE								
Cerebral Cortex	0.33 (0.03)	0.15 * (0.01)	0.28* (0.06)	0.15 * (0.03)	0.33 (0.02)	1.79 (0.16)	0.67 (0.12)	0.86 (0.14)
Olfactory Bulbs	0.04 (0.01)	0.02 (0.00)	0.02 (0.00)	0.02 (0.00)	0.03 (0.01)	0.04 (0.01)	0.08 (0.01)	0.02 (0.01)
Mesencephalon	0.44* (0.08)	0.95* (0.10)	0.33* (0.04)	0.21* (0.03)	0.33 (0.02)	1.89 (0.18)	0.83 (0.10)	0.57 (0.06)
Diencephalon	0.44 (0.07)	1.35 (0.15)	0.33* (0.03)	0.12* (0.02)	0.28 (0.01)	1.94 (0.17)	0.78 (0.09)	1.00 (0.13)
Cerebellum	0.04 (0.01)	0.03 (0.01)	0.03 (0.00)	0.01 (0.00)	0.03 (0.01)	0.01 (0.00)	0.06 (0.01)	0.05 (0.01)
ANABASINE								
Cerebral Cortex	0.38* (0.02)	0.24* (0.02)	0.33 (0.05)	1.57* (0.18)	1.83 (0.16)	1.00 (0.17)	0.40 (0.12)	0.71 (0.16)
Olfactory Bulbs	0.03 (0.01)	0.02 (0.00)	0.03 (0.01)	0.06 (0.01)	0.08 (0.02)	0.06 (0.02)	0.03 (0.01)	0.02 (0.01)
Mesencephalon	0.28* (0.04)	0.24* (0.01)	0.39 (0.06)	0.43* (0.04)	0.83 (0.09)	1.28 (0.16)	0.39 (0.10)	0.18 (0.04)
Diencephalon	0.28* (0.04)	0.20* (0.06)	0.39 (0.07)	0.57 (0.04)	0.78 (0.06)	1.00 (0.19)	0.44 (0.11)	0.24 (0.03)
Cerebellum	0.03 (0.01)	0.03 (0.01)	0.04 (0.01)	0.03 (0.01)	0.06 (0.02)	0.02 (0.00)	0.01 (0.00)	0.02 (0.01)
3-PYRIDYLACETIC ACID								
Cerebral Cortex	0.39* (0.09)	0.36 (0.03)	0.33* (0.03)	0.36 (0.04)	0.44 (0.05)	0.36 (0.04)	1.39 (0.16)	0.86 (0.14)
Olfactory Bulbs	0.04 (0.01)	0.03 (0.01)	0.05 (0.01)	0.04 (0.01)	0.05 (0.02)	0.03 (0.01)	0.06 (0.01)	0.02 (0.00)
Mesencephalon	0.44 (0.06)	0.85* (0.05)	0.39* (0.04)	0.43 (0.06)	0.44 (0.04)	0.36 (0.03)	0.56 (0.07)	0.36 (0.04)
Diencephalon	0.39* (0.03)	1.00* (0.09)	0.33* (0.03)	0.43* (0.05)	0.44 (0.06)	0.36 (0.04)	0.50 (0.04)	0.36 (0.03)
Cerebellum	0.04 (0.01)	0.05 (0.01)	0.05 (0.01)	0.04 (0.01)	0.04 (0.01)	0.04 (0.01)	0.06 (0.02)	0.02 (0.01)

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TREATMENT AND REGION SAMPLED	POST-INJECTION (P.I.) CONDITION							
	ECS 15' P.I.		ECS 45' P.I.		ECS 15' P.I.		ECS 45' P.I.	
	5-HT	5-HIAA	5-HT	5-HIAA	5-HT	5-HIAA	5-HT	5-HIAA
(-) COTININE								
Cerebral Cortex	0.39 (0.04)	0.36* (0.03)	0.33 (0.04)	0.15* (0.02)	0.33 (0.04)	0.43 (0.05)	0.28 (0.03)	0.36 (0.04)
Olfactory Bulbs	0.04 (0.01)	0.03 (0.00)	0.03 (0.00)	0.02 (0.00)	0.03 (0.01)	0.05 (0.01)	0.03 (0.00)	0.05 (0.01)
Mesencephalon	0.44* (0.07)	0.86* (0.07)	0.39 (0.03)	0.15 (0.03)	0.83 (0.05)	0.36 (0.04)	0.33 (0.05)	1.00 (0.12)
Diencephalon	0.44 (0.05)	0.29 (0.04)	0.33 (0.02)	0.12 (0.02)	0.56 (0.04)	0.29 (0.04)	0.28 (0.05)	1.07 (0.11)
Cerebellum	0.04 (0.01)	0.05 (0.01)	0.03 (0.01)	0.01 (0.00)	0.06 (0.01)	0.05 (0.02)	0.03 (0.01)	0.03 (0.01)
MYOSMINE								
Cerebral Cortex	0.39* (0.06)	0.36* (0.04)	0.28* (0.04)	0.86* (0.14)	0.90 (0.19)	0.15 (0.02)	0.44 (0.09)	0.15 (0.03)
Olfactory Bulbs	0.02 (0.00)	0.04 (0.01)	0.03 (0.01)	0.04 (0.01)	0.06 (0.02)	0.01 (0.00)	0.03 (0.01)	0.01 (0.01)
Mesencephalon	0.44* (0.07)	0.31* (0.05)	0.28* (0.06)	0.57* (0.09)	0.56 (0.14)	0.15 (0.03)	0.38 (0.06)	0.15 (0.04)
Diencephalon	0.39* (0.05)	0.43* (0.06)	0.28 (0.05)	0.57* (0.11)	0.61 (0.10)	0.12 (0.03)	0.28 (0.04)	0.15 (0.03)
Cerebellum	0.04 (0.01)	0.03 (0.01)	0.03 (0.01)	0.04 (0.01)	0.00 (0.00)	0.01 (0.00)	0.03 (0.01)	0.01 (0.00)
SALINE								
Cerebral Cortex	0.38 (0.06)	0.12 (0.03)	0.33 (0.04)	0.15 (0.03)	0.38 (0.06)	1.52 (0.19)	0.39 (0.04)	0.12 (0.02)
Olfactory Bulbs	0.03 (0.01)	0.01 (0.00)	0.03 (0.01)	0.01 (0.00)	0.03 (0.01)	0.01 (0.00)	0.07 (0.02)	0.02 (0.01)
Mesencephalon	0.33 (0.04)	0.12 (0.02)	0.33 (0.05)	0.15 (0.03)	0.33 (0.04)	0.21 (0.02)	0.33 (0.04)	0.18 (0.03)
Diencephalon	0.39* (0.04)	0.15 (0.03)	0.33 (0.06)	0.15 (0.04)	0.33 (0.04)	0.15 (0.03)	0.33 (0.05)	0.41 (0.03)
Cerebellum	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.02 (0.00)	0.03 (0.01)	0.02 (0.01)	0.03 (0.01)	0.02 (0.00)

*p<.01

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#642D GOLDSTEIN

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 13, 1974

Grant application No. 642D

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner, and Jacobson

Subject: Leonide Goldstein, D.Sc., CMDNJ-Rutgers Medical School, Piscataway, N.J.
Continuation application No. 642D
"The 'Chronic Nicotine State' and Anxiety: A Behavioral and Electroencephalographic Analysis of Induced and Spontaneous Hyper-activation in Rats".

History

CTR has supported this investigator since 1968.

The current level of support (without assurance of continuation) is $33,350.

Request

Application No. 642D requests $43,890 for one year only.

As this request is for "continuation", it competes on the same basis as a new application.

Documents Submitted (attached)

1. Application dated 7/31/74.

2. Progress Report No. 4, January 1, 1974 - July 31, 1974.

Comment

Dr. Goldstein has also submitted a new proposal (application No. 1009), which is being sent to the same SAB committee. Outside evaluations of both proposals will be sought.

F.W.N.

FWN:lb
Encs.

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#642D

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

RECEIVED
AUG 12 1974

Application for Research Grant

(Use extra pages as needed)

Date: 7/31/74

1. Principal Investigator (give title and degrees): Leonide Goldstein, D.Sc.
Associate Professor of Psychiatry

Co-Principal Investigator: Judith M. Nelsen, Ph.D.
Instructor in Psychiatry

2. Institution & address:

Department of Psychiatry
Institute of Mental Health Sciences
CMDNJ-Rutgers Medical School
P. O. Box 101
Piscataway, New Jersey 08854

3. Department(s) where research will be done or collaboration provided:

Department of Psychiatry

4. Short title of study:

The "Chronic Nicotine State" and Anxiety: A Behavioral and Electroencephalographic Analysis of Induced and Spontaneous Hyper-activation in Rats.

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: One year

7. Brief description of specific research aims:

Because the present application represents the logical continuation of a line of research which has been developed in our laboratory with the aid of support from The Council, it might be useful to review some of the general considerations underlying our studies and the specific experimental goals already achieved which have led us to our current state and have suggested the paths which we would like to pursue. This background will be brief but a more detailed description may be found in the attached "Progress Report".

Electroencephalographic studies with rabbits and rats in our laboratory have indicated that chronic nicotine treatment results in changes in cortical-limbic-reticular formation relationships which might well modify an organism's functional state particularly in terms of the level and nature of arousal (Bhattacharya and Goldstein, Neuropharmacol. 9: 109-118, 1970 and Nelsen, Pelley, and Goldstein, Res. Comm. Chem. Pathol. and Pharmacol. 5: 694-704, 1973). The mesencephalic reticular activating system has long been implicated in the control of arousal. It has been demonstrated repeatedly that electrical stimulation of the mesencephalic reticular formation can produce cortical EEG activation and also, behavioral

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Item 7 continued

activation, for example, the awakening of a sleeping or sedated animal. In fact, we have in this laboratory recently demonstrated this phenomenon as is described in the attached "Progress Report". However, it is apparent that the reticular formation (RF) is not unique in its capacity to induce and maintain arousal. First, the EEG state of sleep which follows mid-collicular section is not necessarily permanent. Batsel (Electroencephal. Clin. Neurophysiol. 12: 421-430, 1960) and Villablance (Electroencephal. Clin. Neurophysiol. 19: 576-586, 1965) have described patterns of spontaneous EEG arousal followed by alternating states of sleep and wakefulness emerging 10 to 15 days after such surgical lesioning. Further, studies have shown that electrical stimulation of portions of the limbic system, particularly the amygdaloid nucleus and hippocampus can produce cortical EEG activation even in "cerveau isole" preparations (Carli, et al., Arch. Ital. Biol. 103: 725-750, 1965). This implies that arousal may be mediated by structures other than the reticular activating system, specifically limbic structures.

It has been suggested that the arousal mediated by the RF is non-specific or generalized in nature while that mediated by the limbic system allows for more selectively motivated or goal-directed behaviors and, further, that the RF and limbic systems are mutually inhibitory (Routtenberg, Psychol. Rev. 75: 51-80, 1968). The EEG studies conducted in our laboratory have indicated that chronic nicotine treatment induces a state of heightened limbic arousal or relatively greater limbic influence and lesser RF influence in the production of cortical activation (Bhattacharya and Goldstein; Nelsen, Pelley and Goldstein). From this finding, it was predicted that such a chronic nicotine state would be beneficial for performance of behaviors requiring focused attention or arousal and discrete goal-directed responses. Direct behavioral testing of this prediction confirmed that chronic nicotine treatment did improve performance of a visual attention task by rats (Nelsen and Goldstein, Psychopharmacologia 26: 347-360, 1972).

At appropriate current levels, delivery of short trains of electrical current to the RF disrupts on-going conditioned behavior presumably because the induced state is one of generalized over-arousal. If the RF and limbic system are mutually inhibitory, then one could hypothesize that the over-arousal or hyper-stimulated state resulting from increased RF activation could be counter-acted by increased limbic activation. In a preliminary study described at length in the "Progress Report", we have tested this hypothesis and found that nicotine was efficacious as an antagonist of the behavioral disruption resulting from RF stimulation. In effect, nicotine is capable of protecting (putatively, by increasing the level of limbic arousal) against an induced state of generalized, unfocused disruptive arousal (which might be considered an animal analog of anxiety) resulting from RF over-arousal. We are presently investigating nicotine's block of RF over-drive in an

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Item 7 continued

experimental situation which allows the simultaneous measurement of EEG activity and behavior on the visual attention task under conditions of RF electrical stimulation. Arrival at this stage of methodological sophistication has resulted from intensive efforts expended toward the development of appropriate technics and the acquisition of the required instrumentation. Reviewed in the "Progress Report" are the methodological advances made with the aid of past Council support. Important is the fact that we are now capable of characterizing more directly the affects of nicotine on the brain electrical events associated with arousal and with the acquisition and performance of behavioral tasks.

Our present research aims relate directly to the line of investigation reviewed. An underlying aim is to utilize animal models for the purpose of exploring some of the features of chronic nicotine administration which for ethical or procedural reasons, cannot be done effectively in humans. Our results, thus far, indicate that chronic nicotine treatment induces a central state which is optimal for carrying out specific learned, goal-directed behaviors. The recent data suggest that nicotine can protect from the behavioral disruption of electrically-induced RF over-drive. The obvious extension presents itself. The hypothesis is that chronic nicotine treatment should ameliorate the behavioral disruption resulting from spontaneous states of RF over-activation. We propose to test for the consequences of chronic nicotinization and withdrawal in rats separated into low anxiety and high anxiety or emotionality categories on the basis of baseline behavioral measures. If rats classified as highly anxious are in that state because of relative RF predominance in the control of arousal, then chronic treatment with nicotine should render them less sensitive to stress; i.e., they should be less susceptible to behavioral breakdown in anxiety-provoking situations than highly anxious rats not treated with nicotine. We propose to compare the relative effectiveness of the expected protection from behavioral disruption in rats with constitutionally different spontaneous arousal levels and to characterize the cortical and subcortical electroencephalographic indices of their functional states.

It has been suggested that withdrawal from the chronic nicotine state (cessation of habitual smoking in humans) is of itself stressful. One would predict that withdrawal would be far more traumatic for subjects constitutionally more anxious or emotional than for less anxious subjects. We propose to quantify the behavioral and electrophysiological consequences of withdrawal from the chronic nicotine state. Of particular interest will be indications of behaviors which might be interpreted as compensatory following cessation of nicotine. Specifically, by methods described in "Item 9", we intend to investigate differences in food consumption (body weight alterations) and spontaneous preferences for alcohol between the high and low anxiety animals during the withdrawal phase.

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DAYS	PHASE	TEST	Hi Anxiety		L. Anxiety	
			Saline	Nicotine	Saline	Nicotine
1	Testing and classification of all subjects into high and low amplitude categories					
:						
14						
1	Treatment I					
:						
7						
8		Control	X	X	X	X
9		Stress (CAT)	X	X	X	X
10						
11		Control	X	X	X	X
12		Stress (Aud. Stim)	X	X	X	X
13						
14						
15		Control	X	X	X	X
16		Stress (Foot-Shock)	X	X	X	X
17						
18		Control	X	X	X	X
19		Stress (RFS)	X	X	X	X
1	Withdrawal I (Body Wt. Monitoring)	Control	X	X	X	X
2		Control	X	X	X	X
3						
4		Control	X	X	X	X
5		Stress	X	X	X	X
6						
7						
8		Control	X	X	X	X
9		Control	X	X	X	X
10						
11		Control	X	X	X	X
12		Stress	X	X	X	X

(Continued)

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DAYS	PHASE	TEST	Hi Anxiety		Lo Anxiety	
			Nicotine	Saline	Nicotine	Saline
1	TREATMENT II					
7						
8		Repeat test	X	X	X	X
9		schedule run	X	X	X	X
10		for TREATMENT				
11		PHASE I	X	X	X	X
12			X	X	X	X
13						
14						
15			X	X	X	X
16			X	X	X	X
17						
18			X	X	X	X
19			X	X	X	X
1	WITHDRAWAL II (ETHANOL PREFERENCE)	Repeat test	X	X	X	X
2		schedule run	X	X	X	X
3		for WITHDRAWAL				
4		PHASE I	X	X	X	X
5			X	X	X	X
6						
7						
8			X	X	X	X
9			X	X	X	X
10						
11			X	X	X	X
12			X	X	X	X

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8. Brief statement of working hypothesis:

The state of arousal, its level and/or nature, is a critical feature in determining the direction and effectiveness of behavior. Chronic nicotine treatment produces shifts in the balance between reticular formational and limbic influences on arousal, yielding a state of enhanced "motivational (limbic) arousal" and reduced "drive (reticular) arousal". This brain functional state is the appropriate one for engaging in focused, goal-directed behavior. Chronic nicotine treatment should counter-act the behaviorally disruptive effects of induced and spontaneous states of reticular over-arousal.

9. Details of experimental design and procedures (append extra pages as necessary)

The experimental procedures which are followed for the preparation of animals with chronic recording and stimulating electrodes, for recording and analysing brain electrical activity, for stimulating discrete brain areas, and for training and testing animals on the visual attention task have been described in previous proposals to The Council. These procedures are included in the attached "Progress Report" and will not be repeated here. However, there are two procedural conventions which we have adopted in past studies of nicotine in the rat and which will be incorporated in future studies. The first concerns the choice of an appropriate dose level. Our studies have always been aimed at investigating behavioral and electrophysiological effects of nicotine which might relate to and, in fact we would hope, lead to a better understanding of the mechanisms of arousal and of the motivations accounting for the widespread self-administration of nicotine by humans. Accordingly, we have chosen dose levels which are considered behavioral (not toxicological) and which co-respond to levels attained when nicotine is self-administered by smokers. The second convention is really related to the first, because it involves the dosage regime and testing schedule. Humans smoke chronically, so that it seems most reasonable that electrophysiological and behavioral information collected in subhuman species will be most meaningful for extrapolation if the chronic, not the acute effects of nicotine are the focus. Further, it has been clearly demonstrated by studies in this laboratory that the effects of acute nicotine administration are very different from those of chronic administration both in terms of behavioral and electroencephalographic responses. Some of these differences and the explicit importance of an awareness of them are described in the attached manuscript of a paper presented at the 1974 meeting of the Behavioral Pharmacology Society. The point is that our studies are designed to elucidate chronic treatment effects.

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It has been demonstrated that high and low emotionality strains of rats will respond differentially to treatment with various psycho-active drugs (e.g., Gupta and Holland, Neuropharmacology 11: 31-38, 1972). Further, within a strain which has not been specially bred for emotionality levels,

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Item 9 continued

it is possible to separate out more highly emotional or anxious animals from low anxiety animals. These subgroups respond differentially to drugs according to behavioral measures. Recently, a method of classifying rats as having high emotionality or low emotionality according to their initiation of consummatory behavior in an open field has been described (Mollenauer, Plotnik and Snyder, Pharmacology, Biochemistry and Behavior 1: 509-514, 1973). This test situation seems to be eminently appropriate for exploring some of the effects of nicotine which should be characterized. Briefly, rats are food deprived for 23 hours before being tested in a circular arena which is marked off in concentric circles and spokes. In the center of the area is a mesh cylinder which can serve as a cat enclosure for stress tests. Four drinking cups are placed, equally spaced, five cm. away from the central mesh enclosure. These cups contain a 32% sucrose solution. Rats are classified for their constitutional level of emotionality according to their readiness to approach and to consume the sweetened water. A pilot study will be run in our laboratory, but other investigators have found it effective to label rats which have not initiated drinking before the fifth testing day (5 minute tests each day) as high emotionals and those which have initiated drinking as low emotionals. The measures which serve as indices of relative levels of anxiety are: (1) cumulative drinking time; (2) approach or cumulative time with the two front feet in the concentric circle nearest the central enclosure; (3) total number of lines crossed; and (4) freezing or cumulative time spent rigidly immobile.

With continued training, differences between groups tend to "wash out", but when exposed to stress or anxiety-provoking stimuli in the test arena, the high anxiety and low anxiety group differences re-emerge. We intend to use this behavioral situation to test for the possible protection provided by chronic nicotine against disruption produced by stressful stimuli and to index the effects of nicotine withdrawal on high and low anxiety subjects. Approximately 50 Holtzman, Sprague-Dawley male rats will be trained and classified as high or low anxiety subjects according to the procedure described above. Members of these two groups will be divided randomly into two subgroups. One of each of the pairs of subgroups will receive chronic nicotine treatment (100 ug/kg, t.i.d., s.c.) and the other chronic saline treatment. The solutions will be coded so that the experimenters will be "blind" insofar as which treatment a subject is receiving. Rats will be maintained on the chronic injection schedule for seven days to insure the development of the chronic nicotine state. Animals will be tested four days a week according to the schedule outlined on the attached table (Treatment Phase I). In each case, a "control test" day will precede a "stress test" day. Rats will be subjected to four types of stress in order to assess the protective efficacy of nicotine as it relates to constitutional or baseline levels of anxiety.

These are:

1. Presence of a cat in the screened core of the experimental apparatus.

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Item 9 continued

2. Aversive auditory stimulation emanating from the screened core of the arena.

3. A short course of unavoidable foot-shock administered in a separate chamber, three minutes before testing in the experimental arena.

4. Reticular formation stimulation delivered to subgroups which will be specially prepared with chronic recording and stimulating electrodes in the brain.

At this point, the injection schedule will be terminated and the Withdrawal Phase I initiated. This phase will be carried out for two weeks. Four test sessions will be conducted each week, three of them control sessions and one, a stress test session. During the period of withdrawal, careful monitoring of body weights will be done. The animals must be partially food deprived to be motivated to drink in the experimental field (23 hours of food deprivation), but a major focus of attention during this phase will be differences in food consumption during the hour of free access to food and water immediately following the test session. In order to discriminate any changes in eating patterns, each animal will be weighed immediately before testing, tested, given free access to food and water for one hour, and then weighed again. Following Withdrawal Phase I, Treatment Phase II will begin. The same treatment and testing schedule followed in the first treatment phase will be repeated, this time with the groups "crossed-over" so that each animal will receive the treatment it had not received during Treatment Phase I. Withdrawal Phase II will follow, during which monitoring of body weights will be done, but additionally, testing will be conducted to discriminate whether differential development of spontaneous ethyl alcohol drinking behavior will be exhibited between treatment and anxiety-level groups.

We are currently investigating the most effective technics to measure tendencies toward spontaneous alcohol consumption and are fortunate in having available the advice of workers at the Center for Alcohol Studies which is located on the same campus as is the Rutgers Medical School.

An important feature of this proposed study is that electroencephalographic measures will be made on certain surgically prepared members of the experimental groups. Because of the logistical prohibitions involved in preparing with chronic brain electrodes and recording from 50 animals, twelve will be chosen out and monitored for electrophysiological effects during the study. Methodological developments in our laboratory (see Progress Report) will allow us to carry out such recording and to further characterize the central nervous system mechanisms operating under conditions of high and low anxiety and the modifications which nicotine provides insofar as these central mechanisms and behavior are concerned.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The laboratory unit is comprized of four specially designed rooms, two of which are 10.5' x 16.5' and two, 9' x 10'. There are also three offices, 10.5' x 12.5'. One of the larger rooms is equipped for behavioral experimentation and the other electroencephalographic recording.

Animal care and maintenance is provided on a per diem basis at the Vivarium of the Rutgers Medical School. A full-time veterinarian supervises the facility.

A Model 1766 Monroe Desk Computer is available for much of the data analysis required for this project. Further, services of the Rutgers University computer are available via a terminal maintained in our building.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append): See attached

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
See attached. Significant reprints are enclosed. More copies of the "review chapter" will be sent when available.

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Leonide Goldstein, D.Sc., P.I.		REDACTED
Judith M. Nelsen, Ph.D., Co-P.I.		
Technical		
Kathleen Pelley, B.A.		REDACTED
Secretary		
	Sub-Total for A	REDACTED
B. Consumable supplies (by major categories)		
Chart and Printer tapes		500.00
Animals (purchase and care)		1,050.00
Chemicals, drugs and electrode equipment		600.00
	Sub-Total for B	2,150.00
C. Other expenses (itemize)		
Travel		500.00
Publication costs		500.00
	Sub-Total for C	1,00.00
	Running Total of A + B + C	37,686,59
D. Permanent equipment (itemize):		
Behavioral modules		400.00
Open field arena		150.00
	Sub-Total for D	550.00
	E	5,652.99
E. Indirect costs (15% of A+B+C)	Total request	$43,889.58

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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5.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
A Study of EEG Correlates of Cognitive Modes	GRS (NIH)	$2,500.00	7/1/74-6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Effect of Microwaves on the EEG and Behavior of Animals	BRH-FDA	$38,000.00	9/1/74-8/31/74

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Mailing address for checks

Principal investigator

Typed Name Leonide Goldstein, D.Sc.

Signature Leon Goldstein Date 7/31/74

Telephone 201 564 - 4416
Area Code Number Extension

Responsible officer of institution:

Typed Name Mr. Harold G. Logan

Title Acting Dean

Signature _____ Date 7/31/74

Telephone 201 564 - 4545
Area Code Number Extension

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[redacted]

Curriculum Vitae

Leonide Goldstein, D.Sc.

Born: REDACTED

REDACTED

1921-35	Student at the Conservatoire National de Musique, Paris
1935	Graduated in violin, harmony and composition
1935-36	R R
1936-37	Technical Assistant Institut de Biologie, Paris
1937-39	Research Assistant
1939-40	R R
1940	Undergraduate studies, University of Paris
1941-42	Research Associate Laboratoire de Physiologie School of Medicine, University of Montpellier, France
1942	Member of the Research Division of the Free French Forces
1942-45	Special assistant to Dr. H.J. Muller, Amherst College, Amherst, Mass.
1944	B.A. and M.A. Amherst College
1945-47	Assistant Professor University of Paris (Physiology and Genetics)
1947-53	Acting Director Laboratoire de Biometrie of the French National Research Council
1951	Doctor of Sciences degree, University of Paris, Sorbonne
1953-58	Assistant Professor, Neurophysiology, Ecole Pratique des Hautes Etudes, Sorbonne.
1958-61	Associate Professor, Pharmacology, Emory Univ., Atlanta, Ga.
1961-64	Neuropharmacologist, Bureau of Research, Neuropharmacology Section, N.J. Neuropsychiatric Institute, Princeton, N.J.
1964-72	Research Scientist Grade 1, Bureau of Research, Neuropharmacology Section, N.J. Neuropsychiatric Institute, Princeton, N.J.
1969-73	Visiting Senior Fellow - Department of Biology - Princeton Univ.
1972	Associate Professor of Psychiatry, Rutgers Medical School, Piscataway, N.J.
1973	Member Graduate Faculty, Rutgers University, New Brunswick, N.J.
1973	Member Psychobiology Area Graduate Program in Psychology, Rutgers University, New Brunswick, N.J.
1974	Member of the Council Eastern Psychiatric Association.

Membership in Scientific Societies:

REDACTED

REDACTED

Honors:

Croix de Guerre (1939-40): Medal of the Free French Forces: Palmes Academiques (1950). Associate Editor "Research Communications in Chemical Pathology and Pharmacology." Member Examination Board French University Studies in the U.S.

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Listings:

American Men at Science - Who is Who.

Publications:

Author or co-author of 160 papers and abstracts.

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6/14/74

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PUBLICATIONS

Goldstein, L., Psychotropic drug-induced EEG changes as revealed by the amplitude integration method in "Psychotropic Drugs and the Human EEG". T. Itil, ed. S. Krager, New York (1974) pp. 58-74.

Goldstein, L., and Stoltzfus, N.W. Psychotropic drug-induced changes in interhemispheric EEG amplitude relationships in Agents and Actions 3, 124-132 (1973).

Goldstein, L., and Nelsen, J.M. Some views on the neurophysiological and neuropharmacological mechanisms of storage and retrieval of information in "Memory Transfer of Information", H.P. Zippel, ed. Plenum Publ. Corp., New York (1973) pp. 155-191.

Nelsen, J.M., and Goldstein, L. Chronic nicotine treatment in rats. I. Acquisition and performance of an attention task. Research Comm. Chem. Pathol. Pharmacol., 5, 681-693 (1973).

Nelsen, J.M., Pelley, K., and Goldstein, L. Chronic nicotine treatment in rats. II. Electroencephalographic amplitude and variability changes occurring within and between structures. Research Comm. Chem. Pathol. Pharmacol. 5, 694-704 (1973).

Nelsen, J.M., and Goldstein, L. Acquisition and performance of an attention task by rats subjected to chronic nicotine treatment. Fed. Proc. 32, 817 (1973).

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R: REDACTED MATERIAL

CURRICULUM VITAE

Judith M. Nelsen, Ph.D.

BORN: REDACTED

MARITAL STATUS: REDACTED

1946-60	Primary and secondary studies, public schools of Town of Lake and City of Cudahy, Wisconsin
1960-63	Undergraduate studies, University of Wisconsin-Milwaukee (Letters and Science, Pharmacy)
1963	Laboratory assistantship in bacteriology (University of Wisconsin-Milwaukee)
1963-65	Undergraduate studies, University of Wisconsin- Madison (Pharmacy, Psychology)
1963-65	Research assistantship in physical chemistry (University of Wisconsin-Madison)
1964	Summer research assistantship in physical chemistry (from the U.S. Department of the Army at the University of Wisconsin-Madison)
1965	B.S. (HONORS) degree. University of Wisconsin. Madison, Wisconsin
1965-70	Graduate studies, Boston University School of Medicine, Division of Medical Sciences, Department of Pharmacology an Experimental Therapeutics (Major professor: Conan Kornetsky, Ph.D., Director, Laboratory of Behavioral Pharmacology)
1965-66	Graduate School Research Fellowship
1966-70	Public Health Service Research Fellowships (N.I.M.H.)
1970	Doctor of Philosophy degree. Boston University. Boston, Mass.
1970-72	Post-doctoral appointment. N.J. Bureau of Research in Neurology and Psychiatry. Box 1000, Princeton, N.J.
1973	Senior Scientist. Rutgers Medical School. Department of Psychiatry, Piscataway, N.J.

HONORS: Sophomore Honors (U.W.); Senior Honors (U.W.); Sigma Epsilon Sigma (U.W.); Rho Chi (U.W.); Phi Kappa Phi (U.W.); Sigma Xi (B.U.)

PROFESSIONAL SOCIETY MEMBERSHIPS:

REDACTED

100354478

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PUBLICATIONS

Nelsen, Judith M. and Conan Kornetsky: Single Dose Tolerance to Morphine Sulfate: EEG Changes. The Pharmacologist 10: No. 2, 1968.

Weil, Andrew T., Norman E. Zinberg, and Judith M. Nelsen: Clinical and Psychological Effects of Marihuana in Man. Science 162: 1234-1242, 1968.

Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. The Pharmacologist 13: No. 2, 1971.

Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. Psychopharmacologia 26: 347-360, 1972.

Nelsen, Judith M. and Conan Kornetsky: Morphine-Induced EEG Changes in Central Motivational Systems: Evidence for Single-Dose Tolerance. Fifth International Congress on Pharmacology (Abstracts, p. 166, #993), 1972.

Goldstein, Leonide and Judith M. Nelsen: Some Views on the Neurophysiological and Neuropharmacological Mechanisms of Storage and Retrieval of Information. In: Memory and Transfer of Information (H.P. Zippel, ed.). Plenum Press, New York, 1973, pp. 155-191.

Nelsen, Judith M.: Neurophysiological and Behavioral Consequences of Chronic Nicotine Treatment. In: Drug Addiction, vol.III (J.M. Singh, L.H. Miller, H. Lal, eds.). Futura Publishing Co., Mount Kisco (N.Y.), 1973 (Chapter accepted for publication).

Nelsen, Judith M. and Leonide Goldstein: Chronic Nicotine Treatment in Rats: 1. Acquisition and Performance of an Attention Task. Res. Comm. Chem. Pathol. and Pharmacol. 5: 681-693, 1973.

Nelsen, Judith M., Kathleen Pelley, and Leonide Goldstein: Chronic Nicotine Treatment in Rats: 2. Electroencephalographic Amplitude and Variability Changes Occurring Within and Between Structures. Res. Comm. Chem. Pathol. and Pharmacol. 5: 694-704, 1973.

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PUBLICATIONS

Nelsen, Judith M. and Leonide Goldstein: Acquisition and Performance of An Attention Task by Rats Subjected to Chronic Nicotine Treatment. Fed. Proc. 32: 817, 1973.

Nelsen, Judith M.: Nicotine Tolerance: Electroencephalographic Activation and "Behavioral Depress". Paper before Behavioral Pharmacology Society, May 11, 1974, Columbia, Maryland.

Nelsen, Judith M., Kathleen Pelley and Leonide Goldstein: Protection by Nicotine of Behavioral Disruption Caused by Reticular Formation Stimulation in the Rat. In preparation.

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#1009 GOLDSTEIN

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 13, 1974

Grant application No. 1009

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner, and Jacobson

Subject: Leonide Goldstein, D.Sc., CMDNJ-Rutgers Medical School, Piscataway, N.J.
New application No. 1009
"Behavioral and EEG Analysis of the Short-term Effects of Smoking Cessation".

History

CTR has supported this investigator through grant No. 642, with renewals and continuations, since 1968.

The enclosed application proposes a new venture using human subjects.

Request

Application No. 1009 requests $31,822, for one year only.

Documents Submitted (attached)

1. Application dated 7/31/74.

2. Reprints of the first two papers listed on the "PUBLICATIONS" page following Dr. Goldstein's CV.

Comment

Dr. Goldstein is also applying for continuation of his long term grant, No. 642D. Both proposals are being sent to the same SAB committee, and outside evaluations of both will be sought.

F.W.N.

FWN:lb
Encs.

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#1009

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
AUG 12 1974

Application for Research Grant
(Use extra pages as needed)

Date: 7/31/74

1. Principal Investigator (give title and degrees): Leonide Goldstein, D.Sc.
Associate Professor of Psychiatry

Co-Principal Investigator: Judith M. Nelsen, Ph.D.
Instructor

2. Institution & address: Department of Psychiatry
CMDNJ-Rutgers Medical School
Piscataway, New Jersey 08854

3. Department(s) where research will be done or collaboration provided:

Department of Psychiatry and Institute for Mental Health Sciences

4. Short title of study:

Behavioral and EEG Analysis of the Short-term Effects of Smoking Cessation

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: One year

7. Brief description of specific research aims: Animal experimentation supported by Grants from the Council on Tobacco Research has revealed that following chronic nicotine administration, animals (rats) become more motivated in certain behavioral features, and are less affected than untreated animals by direct electrical stimulation of the reticular formation, thus apparently less prone to the disruptive effects of what is considered as a model of hyper-stimulation. There are numerous indirect indications of a relationship between hyper-stimulation and anxiety. It appears a reasonable assumption that when deprived of the supposedly protective action of nicotine, there should follow a period of hyper-sensitivity to the disruptive effects of anxiety - provoking events. The specific aims of the study would be to ascertain through both behavioral as well as quantitated brain wave measurements whether habitual tobacco users (cigarette smokers) who discontinue their habit show short-range changes indicative of increased levels of anxiety.

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8. Brief statement of working hypothesis:

Cessation of self-administration of nicotine through chronic cigarette smoking should create a condition of enhanced sensitivity to behaviorally disruptive (i.e., anxiety-provoking) events. The change should be detectable in EEG analysis as well as in simple behavioral performance.

9. Details of experimental design and procedures (append extra pages as necessary)

As can be seen in the procedures and disclosures to the subjects, the study will be divided in two parts. In the first part, EEG recordings and measurements of performance will be obtained on 50 habitual smokers (1 to 1 1/2 packs per day). In order to obtain data untainted by induced anxiety, these subjects will not be told more than that we are seeking to obtain information on their state insofar as brain waves and behavior are concerned. They are to smoke as usual.

After completion of this phase of the study, we will endeavor to convince 10 to 15 of these 50 subjects to stop smoking for a minimal period of two weeks. We feel that the offer of a financial reward should supply sufficient motivation to submit to such a deprivation. In order to insure that the subjects have indeed cooperated, i.e., that they stopped smoking, their respiration will be monitored for CO content. (See below for methodology.)

During the deprivation phase, two or more EEG-behavioral sessions will be held. We hope to obtain by such an approach a meaningful comparison of pre- and post-smoking cessation, since the baseline smoking state will not be different from the routine, everyday state. We also plan to re-record 10 to 15 of the subjects who have not agreed to stop smoking. These will serve as an extra control group.

The EEG will be recorded according to a monopolar montage from the left (L) and right (R) occipital lobes, with the vertex as common reference (O1-Cz; O2-Cz in the International 10:20 system). The electrical activity will be quantitated on-line by electronic integration of amplitudes. For each epoch of measurement (20 sec in the present case) two numerical values will be obtained and automatically listed on paper tape, corresponding to cumulated amplitudes on the L & R electrode locations. From a standard recording session, lasting 10 minutes, thus comprized of 30 epochs, one can compute mean amplitudes per epoch, the time-course variability of the distribution of amplitudes, and measure the degree of relative resemblance in the distribution of the L and R

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9. Continued

amplitudes by analyzing the successive L/R ratios. We have developed computer programs for such calculations.

There is abundant previously published data which indicates that when a subject shifts from a state of relaxation to a state of anxiety, two changes are detectable in EEG measurements: 1) a decrease in the variability of the signals, and 2) a decrease in the difference of L/R amplitudes. Such changes are fully documented in the enclosed reprints.

Insofar as behavioral measurements are concerned, we intend to use a custom-built "choice reaction time" apparatus (see: Frankenhaeuser, M., Myrsten, A.L., and Post, B.; Scand. J. Psychol. 11, 237-245, 1970). Two light stimuli (red and green) will be presented automatically in random order. The subjects will be instructed to respond to the signals by pressing a button on the left arm-rest of the chair for the red light, and on the right arm-rest for the green light. Altogether 100 stimuli will be presented with random order sequences, the mean time between occurences being of 10 sec (range 2 to 18 seconds). The scores will be : 1) time-course variations of the reaction time (latency); 2) number of errors concerning use of the correct button; and 3) number of errors for failing to press and/or to press when no signal was present. It is to be expected that as the level of anxiety increases the number of errors type 3 will increase. Sedation, on the other hand, should tend to increase type 1 errors.

The subjects will be administered the "Mood Adjective Check List" (MACL) which is generally accepted as being valid, reliable and sensitive to changes in mood. A shortened version, including 8 factors and 48 adjectives will be used. The list of items is enclosed. More details on the test can be found in the paper of C. Ague published recently in Psychopharmacologia (30, 323-328, 1973).

The expired air will be analyzed insofar as its contents in CO are concerned. This is important since it is well established that the levels of CO are markedly elevated in the expired air of actively smoking vs non-smoking subjects, There is available a relatively simple device for such measurements.

Blood pressure and heart rate measurements will be performed routinely in order to check for possible severe withdrawal effects, and in the case of their presence, to stop the experimentation. However, it is not expected that this will happen.

The subjects will be questioned during the cessation period concerning their eating and drinking habits. A record of their body weight will be kept.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

We have available a specially designed, electrically shielded and sound attenuated room, for EEG recordings. The behavioral task can be performed in the same room or in an adjoining lounge.

An 8-channel Beckman polygraph is presently available (on rental) as well as an 8-channel HF tape recorder. As previously noted, computer facilities are available for data analyses. A terminal is being installed in our building. The Department has just acquired a Power Frequency Analyzer which is available in case we want to look into EEG frequencies.

11. Additional facilities required:

None except for the continuation of the rental of the polygraph and the purchase of a device to analyze respiration and to measure reaction time.

12. Biographical sketches of investigator(s) and other professional personnel (append): See enclosed.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See enclosed.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested).	% time	Amount
Leonide Goldstein, D.Sc., P.I.		REDACTED
Judith M. Nelsen, Ph.D. Co-P.I.		
EEG Technician (to be recruited)		
Second-year Graduate Student		
Secretary		
Technical		
Sub-Total for A		R
B. Consumable supplies (by major categories)		
EEG chart paper		200.00
Computer time		200.00
Rental EEG machine		4,821.84
Sub-Total for B		5,221.84
C. Other expenses (itemize)		
Fees to subjects 50 @ $5.00 (Baseline)		250.00
10 @ 50.00 (Cessation of smoking)		500.00
Travel funds		500.00
Publication costs		500.00
Sub-Total for C		1,750.00
Running Total of A + B + C		25,323.62
D. Permanent equipment (itemize)		
Ecolyzer		1,200.00
Behavioral modules		1,500.00
Sub-Total for D		2,700.00
E. Indirect costs (15% of A+B+C)		3,798.54
Total request		$31,822.16

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
A Study of EEG Correlates of Cognitive Modes	GRS (NIH)	$2,500.00	7/1/74-6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Effect of Microwaves on the EEG and Behavior of Animals	BRH-FDA	$8,000.00	9/1/74-8/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Leonide Goldstein, D.Sc.

Signature Leonide Goldstein Date 7/31/74

Telephone 201 (Area Code) 564 (Number) - 4416 (Extension)

Check payable to

Mailing address for checks

Responsible officer of institution

Typed Name Mr. Harold G. Logan

Title Acting Dean

Signature _____ Date 7/31/74

Telephone 201 (Area Code) 564 (Number) - 4545 (Extension)

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C. Ague

Table 1. Adjectives of the MACL

Factor A (Aggression)	angry, annoyed, fed up irritable, frustrated, touchy
Factor B (Anxiety)	fearful, jittery, startled anxious, tense, nervous
Factor C (Surgency)	witty, carefree, playful lively, vivacious, cheerful
Factor D (Pleasantness)	refreshed, pleased, lighthearted relaxed, happy, joyful
Factor E (Concentration)	concentrating, thoughtful, serious attentive, introspective, absorbed
Factor F (Fatigue)	tired, bored, drowsy uninterested, weary, distracted
Factor K (Vigour)	active, vigorous, energetic stimulated, quick, alert
Factor H (Sadness)	insecure, sad, downhearted listless, low, gloomy

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R: REDACTED MATERIAL

[redacted]

Curriculum Vitae

Leonide Goldstein, D.Sc.

REDACTED REDACTED

1921-35	Student at the Conservatoire National de Musique, Paris
1935	Graduated in violin, harmony and composition
1935-36	R R
1936-37	Technical Assistant Institut de Biologie, Paris
1937-39	Research Assistant
1939-40	R R
1940	Undergraduate studies, University of Paris
1941-42	Research Associate Laboratoire de Physiologie School of Medicine, University of Montpellier, France
1942	Member of the Research Division of the Free French Forces
1942-45	Special assistant to Dr. H.J. Muller, Amherst College, Amherst, Mass.
1944	B.A. and M.A. Amherst College
1945-47	Assistant Professor University of Paris (Physiology and Genetics)
1947-53	Acting Director Laboratoire de Biometrie of the French National Research Council
1951	Doctor of Sciences degree, University of Paris, Sorbonne
1953-58	Assistant Professor, Neurophysiology, Ecole Pratique des Hautes Etudes, Sorbonne.
1958-61	Associate Professor, Pharmacology, Emory Univ., Atlanta, Ga.
1961-64	Neuropharmacologist, Bureau of Research, Neuropharmacology Section, N.J. Neuropsychiatric Institute, Princeton, N.J.
1964-72	Research Scientist Grade 1, Bureau of Research, Neuropharmacology Section, N.J. Neuropsychiatric Institute, Princeton, N.J.
1969-73	Visiting Senior Fellow - Department of Biology - Princeton Univ.
1972	Associate Professor of Psychiatry, Rutgers Medical School, Piscataway, N.J.
1973	Member Graduate Faculty, Rutgers University, New Brunswick, N.J.
1973	Member Psychobiology Area Graduate Program in Psychology, Rutgers University, New Brunswick, N.J.
1974	Member of the Council Eastern Psychiatric Association.

Membership in Scientific Societies:

REDACTED

Honors:

Croix de Guerre (1939-40): Medal of the Free French Forces: Palmes Academiques (1950). Associate Editor "Research Communications in Chemical Pathology and Pharmacology." Member Examination Board French University Studies in the U.S.

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Listings:

American Men at Science - Who is Who.

Publications:

Author or co-author of 160 papers and abstracts.

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PUBLICATIONS

oldstein, L., Psychotropic drug-induced EEG changes as revealed by the amplitude integration method in "Psychotropic Drugs and the Human EEG". T. Itil, ed. S. Krager, New York (1974) pp. 58-74.

Goldstein, L., and Stoltzfus, N.W. Psychotropic drug-induced changes in interhemispheric EEG amplitude relationships in Agents and Actions 3, 124-132 (1973).

Goldstein, L., and Nelsen, J.M. Some views on the neurophysiological and neuropharmacological mechanisms of storage and retrieval of information in "Memory Transfer of Information", H.P. Zippel, ed. Plenum Publ. Corp., New York (1973) pp. 155-191.

Nelsen, J.M., and Goldstein, L. Chronic nicotine treatment in rats. I. Acquisition and performance of an attention task. Research Comm. Chem. Pathol. Pharmacol., 5, 681-693 (1973).

Nelsen, J.M., Pelley, K., and Goldstein, L. Chronic nicotine treatment in rats. II. Electroencephalographic amplitude and variability changes occurring within and between structures. Research Comm. Chem. Pathol. Pharmacol. 5, 694-704 (1973).

Nelsen, J.M., and Goldstein, L. Acquisition and performance of an attention task by rats subjected to chronic nicotine treatment. Fed. Proc. 32, 817 (1973).

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CURRICULUM VITAE

Judith M. Nelsen, Ph.D.

BORN: REDACTED

MARITAL STATUS: REDACTED

1946-60	Primary and secondary studies, public schools of Town of Lake and City of Cudahy, Wisconsin
1960-63	Undergraduate studies, University of Wisconsin-Milwaukee (Letters and Science, Pharmacy)
1963	Laboratory assistantship in bacteriology (University of Wisconsin-Milwaukee)
1963-65	Undergraduate studies, University of Wisconsin- Madison (Pharmacy, Psychology)
1963-65	Research assistantship in physical chemistry (University of Wisconsin-Madison)
1964	Summer research assistantship in physical chemistry (from the U.S. Department of the Army at the University of Wisconsin-Madison)
1965	B.S. (HONORS) degree. University of Wisconsin. Madison, Wisconsin
1965-70	Graduate studies, Boston University School of Medicine, Division of Medical Sciences, Department of Pharmacology an Experimental Therapeutics (Major professor: Conan Kornetsky, Ph.D., Director, Laboratory of Behavioral Pharmacology)
1965-66	Graduate School Research Fellowship
1966-70	Public Health Service Research Fellowships (N.I.M.H.)
1970	Doctor of Philosophy degree. Boston University. Boston, Mass.
1970-72	Post-doctoral appointment. N.J. Bureau of Research in Neurology and Psychiatry. Box 1000, Princeton, N.J.
1973	Senior Scientist. Rutgers Medical School. Department of Psychiatry, Piscataway, N.J.

HONORS: Sophomore Honors (U.W.); Senior Honors (U.W.); Sigma Epsilon Sigma (U.W.); Rho Chi (U.W.); Phi Kappa Phi (U.W.); Sigma Xi (B.U.)

PROFESSIONAL SOCIETY MEMBERSHIPS:

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PUBLICATIONS

Nelsen, Judith M. and Conan Kornetsky: Single Dose Tolerance to Morphine Sulfate: EEG Changes. The Pharmacologist 10: No. 2, 1968.

Weil, Andrew T., Norman E. Zinberg, and Judith M. Nelsen: Clinical and Psychological Effects of Marihuana in Man. Science 162: 1234-1242, 1968.

Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. The Pharmacologist 13: No. 2, 1971.

Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. Psychopharmacologia 26: 347-360, 1972.

Nelsen, Judith M. and Conan Kornetsky: Morphine-Induced EEG Changes in Central Motivational Systems: Evidence for Single-Dose Tolerance. Fifth International Congress on Pharmacology (Abstracts, p. 166, #993), 1972.

Goldstein, Leonide and Judith M. Nelsen: Some Views on the Neurophysiological and Neuropharmacological Mechanisms of Storage and Retrieval of Information. In: Memory and Transfer of Information (H.P. Zippel, ed.). Plenum Press, New York, 1973, pp. 155-191.

Nelsen, Judith M.: Neurophysiological and Behavioral Consequences of Chronic Nicotine Treatment. In: Drug Addiction, vol.III (J.M. Singh, L.H. Miller, H. Lal, eds.). Futura Publishing Co., Mount Kisco (N.Y.), 1973 (Chapter accepted for publication).

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Nelsen, Judith M., Kathleen Pelley, and Leonide Goldstein: Chronic Nicotine Treatment in Rats: 2. Electroencephalographic Amplitude and Variability Changes Occurring Within and Between Structures. Res. Comm. Chem. Pathol. and Pharmacol. 5: 694-704, 1973.

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PUBLICATIONS

Nelsen, Judith M. and Leonide Goldstein: Acquisition and Performance of An Attention Task by Rats Subjected to Chronic Nicotine Treatment. Fed. Proc. 32: 817, 1973.

Nelsen, Judith M.: Nicotine Tolerance: Electroencephalographic Activation and "Behavioral Depress". Paper before Behavioral Pharmacology Society, May 11, 1974, Columbia, Maryland.

Nelsen, Judith M., Kathleen Pelley and Leonide Goldstein: Protection by Nicotine of Behavioral Disruption Caused by Reticular Formation Stimulation in the Rat. In preparation.

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Smoking Cessation

This study will be divided into two separate phases. During the first phase, EEG recordings, behavioral measurements and respiratory gas exchanges will be done on fifty habitual smokers (one pack to one and one half packs a day). The subjects will be told exactly what the study is about, namely to obtain information on the habitual state of a smoker. A widely used questionnaire relative to state of anxiety, the "MALL", will be filled out by the subjects (see enclosed instructions and disclosures before obtaining consent). A simple questionnaire on smoking habits will also be filled out (copy enclosed).

In the second phase, the subjects will be recalled and offered treatment to discontinue for two to three weeks their habit. The ones interested will be briefed on the procedures to be used. They will be recorded twice before treatment and twice after cessation of smoking. Although the measurement procedures to be used will be the same as used during the first phase, they will nonetheless be repeated carefully and completely. A separate consent form will be obtained.

The reason for the two distinct phases is that changes in the level of anxiety can only be detected in relation to "normal" daily routines. It is likely that the baseline measurements obtained one week or so before cessation of smoking, when the subjects know that they will have to go through the procedures, will give a somewhat distorted baseline to which to compare post-smoking cessation data.

It should be pointed out that no pressure will be applied to convince smokers to undertake cessation procedures.

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Disclosures to Subjects

Phase I

You are going to be involved in a study of certain physiological parameters in smokers. There is no hidden purposes to this study other than to obtain information on the base state of the smoker, as compared to that of the non-smoker. This is made clear by the questionnaire on smoking habits.

We are going to measure four things on you: Your EEG (brain waves), your reaction time, your respiratory exchanges insofar as gases are concerned, and finally your mood through a simple paper and pencil questionnaire. Let me explain briefly what these procedures are, what data they provide and what risks (in fact there are none) to your health and well being.

a) EEG Recordings. As you may know, the brain produces continuously varying electrical potentials which can be picked up and recorded on paper, yielding patterns of the variations of potentials in time. The EEG procedure is harmless and produces little or no discomfort. The electrodes (i.e., the units which pick up the electrical activity) are small gold-plated cups. These are put in contact with the scalp via a paste containing electrolytes. It is not exaggerated to say that the EEG has been recorded on millions of people and not a single adverse reaction has been reported. The paste is neutral and will not produce any skin reaction, even in poly-allergic subjects. Keep in mind two important things. First, the EEG machine is strictly a one-way machine insofar as electrical currents are concerned. It accepts an input but has no other output than to the recording oscillographs and their attached ink-pens. The electrode wires are one-way conductors. The EEG works like the electrocardiogram. Second, EEG patterns cannot tell anything covering your thoughts. All they tell us is the relative functional state of your brain at the time the recording is performed. The EEG is like the envelope containing a letter. By looking at the envelope we can say from where it was mailed, to whom it is addressed and how long it travelled; by the weight we can infer how much writing it contains. But that is all. We cannot say what is in the letter. So, do not worry that we will know what is on your mind. You can think of EEG recordings as wire-taping of the brain. We are, in effect, listening to what is going on in your brain. However, we do not listen to one conversation going on between two persons but an astronomical number of conversations between an astronomical number of persons going on at the same time. What we hear is something like the noise which would be generated by the population of New York City crowded into Shea Stadium with everybody talking. Since we have no way of

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unscrambling these conversations, we cannot conceivably understand their contents even in the remotest way. What we can establish, however, is knowledge on the magnitude of the participants, the speed at which they talk, the loudness of their voices, and how concordant or discordant is their conglormerated vocalization. But this is all we can get at present and probably for a long time to come. What we try to find out is whether the amount of conversation going on is the same in smokers and in non-smokers.

As soon as the recording is over (10-15 minutes), we will remove the electrodes and we promise to do a good job, so that no traces of the paste will remain.

b) Reaction Time. This is a simple procedure which permits us to measure how attentive to a task you are, and how fast you get tired. You will sit in front of a panel on which two lights will appear, either a red light or a green light. On the arm rests of the chairs are two levers. All we ask you to do is to press on the left arm lever when you see the red light and on the right arm lever when you see the green light. The signals will appear in a random fashion so you cannot forecast when the red has appeared whether the next presentation will be red or green, neither can you guess how regular or irregular the presentations will be. Try to press as quickly as you can.

c) Respiratory Gases. You will be asked to blow into a tube, so that we can determine the level of carbon monoxide in your breath. This information is needed to establish how smoking affects your respiratory exchanges. It has nothing to do with pathology.

d) Questionnaire. This is part of the inquiry into your personality. As you can see the questions are not meant to yield the kind of information which could be used against you. However, you can rest assured that all you say will be treated in strict, absolute confidentiality.

In a general way, we intend to respect your personality in the most careful way. For us, you will be a number of a certain age, weight and height. That is all. Your name will not be disclosed unless you speciffcally request it. We have a safe for storing information; the safe is located in a room with a lock which can only be opend by Dr. Goldstein and Security Officers.

Finally, you are free to discontinue your participation at anytime.

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Phase II

Since you are interested in discontinuing your smoking habits, we are inviting you to participate in the same studies of physiological parameters as you did previously but this time in connection with the procedures which should help you "kick the habit".

Read Disclosures for Phase I starting with the second paragraph.

Insofar as procedures for discontinuation they are as follows:

You will simply stop smoking. One of the most widely used method for counteracting the absence of cigarettes is to chew gum or to suck candies. Some ppople find comfort in keeping a toothpick in their mouth. You are free to use any means you want to help you go through the period without cigarettes. Do not use so-called "replacement" products such as Nicoban.

You should not be unduly worried about the effects of withdrawal from tobacco. You may become irritable or unduly nervous. However, as evidenced by a large amount of data, you should not be made really ill; uncomfortable, yes; ill, no. Again, if you cannot stand it, the solution is simple: resume smoking. Go slowly at it, that is all. If you should feel sleepy, use coffee--moderately, of course.

We intend to obtain two sets of measurements before you undergo the treatment, and one and two weeks following smoking cessation.

Again, you can discontinue your participation at any time. If you complete the procedure, you will receive a $50.00 fee. If you dropout, you will be rewarded at the rate of $5.00 per session attended.

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Smoking Cessation Questionnaire

Code #_____ Date_____

Age_____ Sex_____ Height_____ Weight_____

Right_____ or left_____ handed.

How long have you been smoking?_____

How did you start?_____

_____

Have you ever tried to stop? _____Yes _____No.

If yes, how many times?_____ How long?_____

How many cigarettes do you use per day (average)?_____

What brand?_____

When do you smoke the first cigarette of the day?_____

When do you smoke the last cigarette of the day?_____

How long before this recording did you last smoke?_____

Did you experience any unusual event today?_____Yes _____No

If yes, explain_____

_____

Did you experience any unusual event yesterday? _____Yes _____No

If yes, explain_____

_____

How would you judge today insofar as your performance is concerned?

above average_____ average_____ below average_____

Do you use alcoholic beverages? _____Yes _____No

If yes, could you indicate (roughly) your weekly consumption:

_____beer _____wine _____whiskey _____liquor _____other

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Age Sex Height Weight

As best you can remember, list the main illnesses up until now.

a:

Any surgical operations? Yes No. If yes, which?

When

How did you sleep last night? as usual better worse

Characterize in any way you want, what effect or effects you obtain from smoking.

Are you taking any medication either on prescription or over-the-counter, such as anti-histamines or aspirin? Yes No

If yes, which

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#994 - HILTON

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THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

July 18, 1974

Grant application No. 994

PHARMACOLOGY

To: The committee comprising Drs. Bing, Jacobson and Liebow

Subject: James G. Hilton, Ph.D., University of Texas Medical Branch
New application No. 994
"Influence of the CNS Upon Ganglion Stimulation".

History

A preliminary inquiry was handled as Case No. 269; the Executive Committee voted to encourage formal proposal.

Request

Application No. 994 requests $21,902, plus two additional years.

Documents Submitted

Attached is undated application received by CTR on July 15, 1974.

Reprints of publications listed in the application as numbers 1 through 5 were provided and will be forwarded if you wish.

Comment

We shall doubtless seek appraisal of this request by outside consultants.

F.W.N.

FWN/meb
Encl.

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#G9U

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 15 1974

Application for Research Grant (Date:

(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

James G. Hilton, Ph.D.
Professor of Pharmacology and Toxicology

2. Institution & address:

University of Texas Medical Branch
Galveston, Texas 77550

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology and Toxicology

4. Short title of study:

Influence of the CNS Upon Ganglion Stimulation

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

It has been demonstrated that the section of the spinal cord at C-8 causes a marked decrease in the pressor response elicited by the intravenous administration of nicotine. The explanation offered for this reduction in responsiveness was that the nicotine pressor response was due to the stimulatory actions of nicotine at both the peripheral ganglia and the central vasomotor center and that section of the cord blocked the peripheral pathway of the central vasomotor stimulation. The reduction of nicotine pressor response following removal of CNS influence by total spinal anesthesia has been confirmed in this laboratory. In addition, preliminary studies using the nicotine like ganglion stimulant 1, 1-dimethyl-4-phenylpiperazinium (DMPP) and a technique of close intra-arterial injection have shown that removal of CNS influence by total spinal anesthesia markedly reduces the localized ganglion stimulating actions of this drug. The study proposed in this application is aimed at confirming these preliminary observations and at determining the effects of removal of CNS influence upon other ganglion stimulant agents. Since the early observations involved nicotine rather than DMPP it is important to determine whether or not the localized ganglion stimulation of this agent is also depressed by total spinal anesthesia and by section of the spinal cord. Also studies will be conducted using muscarinic ganglion stimulants such as 4-(m-chloro-phenylcarbamoyloxy)-2-butynyl-trimethyl-ammonium chloride (McN-A-343) and neostigmine, the natural chemical transmitter acetylcholine, and electrical stimulation of the preganglionic nerve trunk.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

8. Brief statement of working hypothesis:

The hypothesis being tested in these studies is that the central nervous system exerts a modulating influence upon the autonomic ganglia and that removal of this influence will decrease the ability of chemical agents to stimulate the ganglia.

9. Details of experimental design and procedures (append extra pages as necessary)

Experimental Design and Procedures

Dogs anesthetized with sodium pentobarbital will be used as the experimental animals. The trachea, femoral artery and jugular vein will be exposed and cannulated. Artificial respiration will be maintained by the use of a Harvard pump. Arterial pressure will be monitored from the femoral artery. Through a lateral incision in the left thorax the region of the left stellate and caudal cervical ganglia will be located and exposed by blunt dissection. The left omocervical and axillary arteries will be located and ligated. The internal thoracic artery will be located and cannulated with polyethylene tubing. (See figure 1, Graca, Hilton and Silva, reprint enclosed, for anatomical relationships in this area). A modified Walton-Brodie Strain Gauge Arch will be placed on the left ventricle and cardiac force of contraction will be recorded on an appropriate polygraph. In those experiments where electrical stimulation of the preganglionic nerves is desired the left sympathetic paravertebral chain will be located between T2-T3 and appropriate electrodes will be placed on the nerve chain at this point. In all experiments the left vagus will be severed in the mid-cervical region. All injections of ganglion stimulating drugs will be made into the internal thoracic artery. Cardiac force of contraction will be measured in arbitrary units and increases or decreases in force resulting from drug administrations will be recorded and reported as percent change (see figures 5 and 6, Garca, Hilton and Silva). Spinal anesthesia will be produced by the intrathecal injection of 20 ml of 1.5% piperocaine hydrochloride at L-8. Spinal section will be carried out by severing the spinal cord at C-6.

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9. continued

Using the technique described several series of experiments are planned.

(1) To confirm the prior observations and to develop a knowledge of the dose-response relationships, graded doses of 1,1-dimethyl-4-phenylpiperazinium (DMPP) ranging from 1 to 100 micrograms will be administered by close intra-arterial injection before and after total spinal anesthesia.

(2) To determine whether or not there are differences between nicotine and DMPP Series 1 will be repeated using graded doses of nicotine.

(3) To determine whether or not the depression of responsiveness is due to blockade of a central nervous system influence or a possible selective receptor blockade by the piperocaine, two series of experiments will be carried out. In one series the cord will be sectioned before and after the graded doses of nicotinic ganglion stimulant drug and in the other series piperocaine will be administered intravenously rather than by the intrathecal route.

(4) To determine whether or not the depression of responsiveness extends to preganglionic electrical stimulation Series 1 and 2 will be repeated using supramaximal electrical stimulation of the sympathetic nerve trunk.

(5) To determine whether or not the depression of responsiveness extends to muscarinic ganglion stimulant drugs the series will be repeated using graded doses of 4-(m-chloro-phenylcarbamoyloxy)-2-butynyl-trimethylammonium chloride (McN-A-343) a specific muscarinic ganglion stimulating drug.

(6) To determine whether or not the depression of responsiveness extends to the proposed natural chemical transmitter the series will be repeated using graded doses of acetylcholine.

(7) If time permits these studies will be extended to include studies on the effects of removal of the influence of the central nervous system upon the blocking actions of nicotinic and muscarinic ganglion blocking agents.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The principal investigator has approximately 500 sq ft of laboratory space available for conducting the research outlined in this project. The space is equipped with the major pieces of equipment required to carry out the proposed research. These include a Grass Model 5 polygraph with appropriate preamplifiers and transducers, a twin viso polygraph, respiration pumps and an Epic 3000 programmed calculator. In addition to these items the Department of Pharmacology is well equipped with a variety of physiological equipment Hewlett Packard Calculator.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

12. Biographical sketch

James Gorton Hilton, Ph.D.

Date of Birth: REDACTED
Place: REDACTED
Marital Status: REDACTED

Education:

Virginia Polytechnic Institute, B. S., 1947, Major: Chemistry, Minor: Mathematics
University of Tennessee, M. S., 1952. Major: Pharmacology, Minors: Physiology and Biochemistry
University of Tennessee, Ph.D., 1954. Major: Pharmacology, Minors: Physiology and Biochemistry

Experience:

University of Virginia: Research Associate in Pharmacology, 1948-1950.
University of Tennessee: Teaching Fellow in Pharmacology, 1950-1953.
University of Mississippi: Assistant Professor of Pharmacology, 1953-1955; Associate Professor of Pharmacology, 1955-1958.
Marquette University: Associate Professor of Pharmacology, 1959-1961.
University of Texas Medical Branch: Associate Professor of Pharmacology 1961-1963. Professor of Pharmacology, 1963-Present.
Interim Chairman of Pharmacology 1964-1965.
Acting Co-Ordinator of Sponsored Research, 1967-1968.

Societies:

REDACTED

REDACTED

Additional Information:

REDACTED

Fulbright Lecturer in Pharmacology, Department of Pharmacology, University of San Agustin, Arequipa, Peru, 1959-60.
Consultant to A.M.A. Council on Drugs for New and Non-Official Drugs, 1963-66.

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12. Continued

Pharmacology and Endocrinology Fellowships Review Panel, NIH, 1964-67.

Committee on Persistent Pesticides of the National Research Council, 1967-1969.

Fulbright Scholar in Pharmacology, Department of Pharmacology, Centro de Biologia, Instituto Gulbenkian de Ciencia, Oeiras, Portugal, 1968-69.

Consultant, Pesticide Regulation Division, Environmental Protection Agency 1970-72.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

13. Publications

1. Graca, A. S., Hilton, J. G., and Silva, A. J.: Effects of Muscarinic Ganglionic Stimulation and Blockade upon Ganglion Responses to DMPP and Nicotine. Arch. inter. de Pharmacodyn. 186: 379-387, 1970.

2. Hilton, J. G.: Effects of Various Drugs upon the Neostigmine Pressor Response. Arch. int. de Pharmacodyn. et de Therap. 171: 116-121, 1968.

3. Steinberg, M. and J. G. Hilton: Effect of Sympathectomy and Adrenalectomy upon Ganglion Blockade. J. Pharmacol. and Exptl. Therap. 156: 215-220, 1967.

4. Hilton, J. G. and M. Steinberg: Effects of Ganglion and Parasympathetic Blocking Drugs upon the Pressor Response Elicited by Elevation of the Intracranial Fluid Pressure. J. Pharmacol. and Exptl. Therap. 153: 285-291, 1966.

5. Fenner, P. A. and J. G. Hilton: The Effects of Neostigmine upon Ganglion Responses after Administration of Blocking Drugs. Brit. J. Pharmacol. & Chemotherapy. 21: 323, 1963.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
James G. Hilton		REDACTED
Technical		
Mary Pat Kelly		REDACTED (Includes Staff Benefits)
To be recruited		
Sub-Total for A		R
B. Consumable supplies (by major categories)		
Animals		2,000
Drugs and Chemicals		500
Surgical Equipment and Supplies		600
Recording Supplies		650
Sub-Total for B		3,750
C. Other expenses (itemize)		
Travel to Federation Meetings		450
Sub-Total for C		450
Running Total of A + B + C		19,045
D. Permanent equipment (itemize)		
None		
Sub-Total for D		
E. Indirect costs (15% of A+B+C)		2,857
Total request		21,902

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	15,350	3,750	450	0	2,933	22,483
Year 3	15,875	3,750	450	0	3,012	23,087

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Planned A study of drugs useful in the treatment of decompression sickness (with Dr. C.H. Wells of the Shrine Burn Institute)	Navy Contract		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name James G. Hilton, Ph.D.

Signature James G. Hilton Date 7/8/74

Telephone 713 (Area Code) 765-1561 (Number) (Extension)

Checks payable to

University of Texas Medical Branch

Mailing address for checks

V. F. Thompson, Vice President for Business and Hospital Affairs
The University of Texas Medical Branch
Galveston, Texas 77550

Responsible officer of institution

Typed Name Dr. Edward N. Brandt, Jr.

Title Dean of Medicine

Signature Edward N. Brandt Jr. Date 7-10-74

Telephone 713 (Area Code) 765-2671 (Number) (Extension)

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#1003 KLAIBER

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 1, 1974

Grant application No. 1003

PHARMACOLOGY

To: The committee comprising Drs. Gardner, Jacobson, and Sommers

Subject: Edward L. Klaiber, M.D., The Worcester Foundation for Experimental Biology, Inc., Shrewsbury, Mass.
New application No. 1003
"Central Nervous System Adrenergic Functioning and Cigarette Smoking".

History

An informal inquiry was handled as case No. 273: The Executive Committee voted to encourage formal application.

Request

Application No. 1003 requests $20,332 for a pilot study, with future requests to be contingent on results obtained during the first year.

Documents Submitted (attached)

1. Application dated July 30, 1974 (14 pages)
2. CV's of Drs. Klaiber, Broverman, and Vogel

F.W.N.

FWN:lb
Encs.

cc: Dr. Stone

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

#1003

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 31 1974

Application for Research Grant
(Use extra pages as needed)

Date: July 30, 1974

1. Principal Investigator (give title and degrees):

Edward L. Klaiber, M.D. Senior Scientist

2. Institution & address:

The Worcester Foundation for Experimental Biology, Inc.
222 Maple Avenue
Shrewsbury, Massachusetts 01545

3. Department(s) where research will be done or collaboration provided:

Non-applicable

4. Short title of study:

Central Nervous System Adrenergic Functioning and Cigarette Smoking

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: One year

7. Brief description of specific research aims:

Brief Rationale

The proposed research is based on the hypothesis that smokers tend to have impaired central adrenergic functioning compared to non-smokers. Impaired central adrenergic functioning has been associated with mental states of fatigue, apathy, reduced motor activity and negative mood states. Cigarette smoking is believed to act as a central adrenergic stimulant thereby alleviating these adverse states.

Impaired central adrenergic functioning may be, in part, secondary to insufficient gonadal hormone stimulation. The gonadal hormones are thought to influence central adrenergic functioning through their ability to inhibit monoamine oxidase (MAO) activity. This enzyme is important in the intra-neural regulation of monoamines that act as neurotransmitters in adrenergic neurons.

Specific Aims

The specific aims of the proposed research are that smokers, compared to non-smokers,

a) have relatively poor central adrenergic functioning as reflected by rate of EEG "driving" responses to photic

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stimulation; and by performances of "automatized" tasks. Both of these indices have been shown to be sensitive to adrenergic stimulants and blocking agents;

b) have relatively high levels of plasma monoamine oxidase (MAO) activity which act to impair central adrenergic functioning.

c) have relatively low levels of free plasma testosterone, and less anthropometric evidence of testosterone stimulation. Testosterone is believed to inhibit MAO activity thereby enhancing central adrenergic activity.

d) In addition, the proposed research would test the hypothesis that smoking a single cigarette will promptly affect the rate of EEG "driving" responses to photic stimulation in a manner similar to an adrenergic stimulant.

The bases for these hypotheses are described in detail below.

Introduction

This research group has been investigating aspects of central adrenergic functioning in humans for the past ten years, primarily in the context of mental depression, but also in relationship to individual differences in normal cognitive functioning, learning disabilities, and, most recently heroin addiction.

Our indices of central adrenergic functioning are:

1) Frequency of EEG "driving" responses to photic stimulation, i.e., the tendency of EEG rhythms to mimic the frequency of a bright flashing light. Adrenergic stimulants such as amphetamine (Shetty, 1971) and norepinephrine (Floru et al., 1962) tend to inhibit this response, while adrenergic blocking agents such as chlorpromazine (Killam et al., 1967) tend to augment the response.

2) Performances of overlearned repetitive "automatized" tasks. Examples of such automatization tasks are speeds of performance of simple addition problems, naming color hues, object naming, etc. These task performances are enhanced by adrenergic stimulants such as amphetamine and impaired by adrenergic blocking agents such as chlorpromazine (Broverman et al., 1968).

3) Plasma monoamine oxidase (MAO) activity. The MAO enzyme plays an important role in the intraneural regulation of monoamines that are believed to act as neurotransmitters in adrenergic nerves (Kopin, 1964).

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While plasma and brain MAO activity may not be the same, we believe that the plasma MAO activity tends to reflect the state of brain MAO activity since both share, to a degree, a common biochemical environment. In support of these assumptions, we have noted that:

A) blood and brain MAO activity are positively correlated in their rate of recovery from an MAO inhibitor (Kobayashi and Kizuka, 1972);

B) in the rat, the jugular venous blood level of MAO activity averages 20% greater than that found in carotid arterial blood, indicating that the brain is one source of blood MAO activity (Kobayashi and Kizuka, 1973);

C) in humans, plasma MAO activity is significantly positively correlated with frequency of EEG "driving" responses to photic stimulation (Klaiber et al., 1971; Vogel et al., 1974); and significantly negatively correlated with performances of "automatization" tasks (Klaiber et al., 1967).

The question of the possible heterogeneous nature of monoamine oxidase has been a source of controversy. The evidence for the existence of multiple forms of MAO has been provided mainly by Sandler's group in England (Youdim and Sandler, 1967; Youdim et al., 1969; Youdim, 1973). Their position has been supported by other workers such as Shih and Eiderson (1973), Coquil et al. (1973), and Gomes et al. (1969). However, Hartman and Udenfriend (1972) have provided evidence to indicate the near-homogeneous nature of MAO based on immunological studies showing, for example, that the 3 MAO isoenzymes isolated by Gomes et al (1969) from beef liver were antigenically identical. In a recent study, Tipton, et al (1973) have shown that the multiple forms of MAO reported by Youdim et al. (1968, 1969, 1973) may be due to the binding of different amounts and/or types of lipid to a single enzyme species. When MAO was partly purified from human brain, that prepared according to the method of Youdim et al. (1968, 1969, 1973) separated into multiple bands whereas that prepared using perchlorate showed only a single band. On the basis of substrate specificities and response to enzyme inhibitors, Tipton et al. (1973) concluded that their findings were consistent with the contention that multiple forms of human MAO arise from differential binding of membrane material to a single enzyme species.

Although this question of the nature of MAO remains unresolved; it has little relevance to the MAO activity measurements of the blood. The plasma assay measures total MAO activity contained per unit volume, irrespective of enzyme origin or type. The amount of MAO activity in the plasma is minute and is probably not, itself, physiologically significant. However, as indicated above, it does appear to be a crude index of brain MAO activity.

Gonadal Steroid Hormones and Central Adrenergic Functioning

Our research has led us to believe that the gonadal steroid hormones, testosterone, estrogen and progesterone, significantly influence central adrenergic functions via their regulatory influence upon MAO activity.

Thus, hypothalamic MAO activity cycles with estrus cycle in the rat (Kamberi and Kobayashi, 1970); and plasma MAO activity cycles with menstrual cycle in humans (Klaiber et al., 1971). In the rat, ovariectomy resulted in elevated hypothalamic MAO activity (Kobayashi et al. 1966); and diminished behavioral activity (Wang, 1923; Richter, 1927); while the administration of

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estrogens acted to return both hypothalamic MAO activity (Kobayashi et al., 1966) and behavioral activity (Young add Fish, 1945) towards normal. In the human, amenorrheic women, who are known to be estrogen deficient (Brown et al., 1969), had abnormally elevated plasma MAO activity (Klaiber et al., 1971a) and heightened EEG driving response rates (Vogel et al., 1971); both of which could be returned to normal by the administration of oral conjugated estrogen.

Male endocrine patients requiring testosterone therapy were found, before treatment, to have elevated plasma MAO activity, which I.M. injections of testosterone returned towards normal (Klaiber et al., 1974). Anthropometric indices of testosterone stimulation, e.g., pubic hair development, chest and biceps circumferences, were found to be positively correlated with performances of automatization tasks (Klaiber et al., 1967). A 3 hour infusion of testosterone significantly enhanced performances of such tasks in normal men (Klaiber et al., 1971b); while injections of testosterone in endocrine patients enhanced both automatization performance and reduced their EEG driving response rates (Stenn et al., 1972).

Psychological Significance of Variations in Central Adrenergic Functioning

The central adrenergic functions have been postulated by Hess (1954) to be "ergotropic" i.e., they are believed to regulate such "work" activities of the brain as wakefulness, alertness, motor activity, sensory reactions, and to be associated with positive mood states. A deficit in central adrenergic functioning, on the other hand, has been postulated by Schildkraut (1964) to be the basis of mental depression whose symptoms include fatigue, apathy, reduced motor activity, and negative mood states. We have reported that depressed female patients have extremely high levels of plasma MAO activity and elevated levels of EEG driving, both of which could be corrected by the administration of oral conjugated estrogen (Klaiber et al., 1971a; 1974).

A Conceptual Model for Addiction

The above observations have led to the following conceptual model. Within the range of normal variation, individuals with relatively less gonadal steroid hormone stimulation tend to have relatively elevated MAO activity and reduced central adrenergic functioning. The negative psychological concomitants of reduced central adrenergic functioning, i.e., fatigue, depression, apathy, etc., then ensue. Such individuals will tend to be attracted to, and come to depend upon, any drug or substance which tends to momentarily alleviate these states, i.e., drugs that act as central adrenergic stimulants, at least in their short-term effects.

Central Adrenergic Functioning and Drug Addicts

Our first test of this hypothesis was a small sample (n=19) of male drug addicts using heroin and other drugs such as marijuana. Heroin is known to affect norepinephrine levels in the brain (Way and Shen, 1971) and marijuana has a predominantly adrenergic effect (Dagirmanjian & Byrd, 1962). The patients were studied while off all drugs. When matched by age with normal controls, the addicts had significantly elevated plasma MAO activity and significantly less pubic hair development. Chest and biceps circumferences were also smaller, but this could be due to the adverse diets that such individuals frequently have. Less ambiguous was the fact that over 40% (eight of the addicts) had histories of gonadal impairment (undescended testes; surgically removed testes; underdeveloped genitalia, etc.) that preceded their use of

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heroin.

The anthropometric data, e.g., low pubic hair ratings, suggests that testosterone activity is reduced in these individuals. A low level of testosterone activity could account for their elevated MAO activity which, in turn, could result in poor central adrenergic functioning. It is our hypothesis that the drugs that these addicts have used, such as heroin and marijuana, stimulate CNS adrenergic functioning and thereby momentarily relieve the negative symptoms associated with deficient CNS adrenergic functioning. This initial study has formed the basis of a research proposal submitted to the U.S.P.H.S.

Central Adrenergic Functioning and Cigarette Smoking

A second pilot study of our hypothesis that impaired central adrenergic functions are associated with addictive tendencies involving cigarette smoking.

Twenty-four subjects, half smokers (1 pack a day or more); half non-smokers; half male; half female; were compared on rates of EEG "driving". Two such trials were obtained, one at least 3 hours after the last cigarette smoked by the smokers; and another trial immediately following the smoking (inhalation required) of one cigarette.

Our results indicate that the smokers have greater rates of EEG driving than non-smokers ($p<.001$); and that smoking one cigarette significantly reduces this rate of EEG driving in all subjects ($p<.001$). Figure 1 portrays these results. The reduction in EEG driving associated with cigarette smoking is probably a direct result of the CNS adrenergic stimulating action of nicotine. Once again, as in the drug addict, addictive individuals, i.e., cigarette smokers, have evidence of adrenergic insufficiency. The smoking condition also indicates that the adrenergic insufficiency tends to be alleviated by cigarette smoking.

Other studies have also demonstrated EEG differences between smokers and non-smokers, (Hauser et al., 1958; Brown, 1973, 1968). Those studies, however, lacked a theoretical orientation within which to interpret the observed relationships. The results of the present study, on the other hand, conform to the expectations derived from our theoretical model involving central adrenergic functioning.

The above pilot study was based on a relatively small number of subjects. We propose to repeat that study, and add additional measures in order to meet the following specific aims.

Specific Aims

Study I

1) To assess the frequency of EEG "driving" responses to photic stimulation of smokers and non-smokers in a larger homogeneous sample of subjects, i.e., 21 to 30 year old males.

2) To assess the plasma MAO activity of the above smoking and non-smoking samples.

3) To assess performances of automatized tasks of the smokers and non-smokers

4) To assess plasma total and free testosterone levels and serum LH and FSH in the above samples.

5) To assess anthropometric indices of testosterone stimulation in the above samples.

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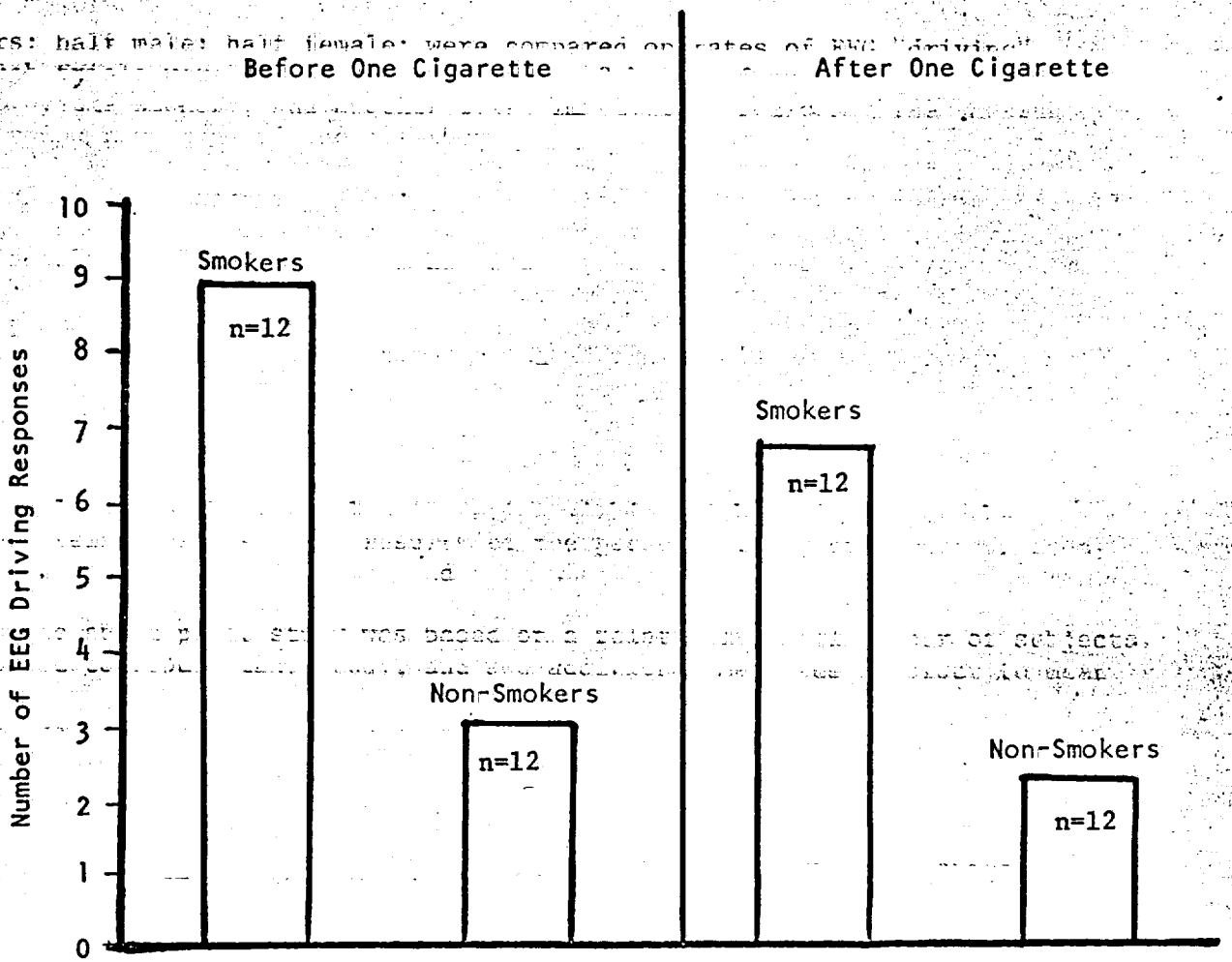

Figure 1

EEG Driving Responses in Smokers and Non-Smokers,

Before and After One Cigarette

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Study II

1) To assess the effects of smoking one cigarette on EEG driving.

In the short time span of this smoking experiment, it is most unlikely that any significant change will occur in blood testosterone levels. Therefore, neither plasma testosterone nor plasma MAO activity will be measured after smoking one cigarette. Ideally, these assumptions should be empirically tested. However, they are eliminated in the present proposal in an effort to minimize costs.

8. Brief statement of working hypothesis:

The working hypotheses of this proposal are:

Study I

1) Smokers will evidence greater frequency of EEG "driving" responses to photic stimulation than non-smokers.

2) Smokers will have higher plasma MAO activity than non-smokers.

3) Male smokers will have less anthropometric evidence of testosterone stimulation than male non-smokers.

4) Male smokers will have lower free, but not necessarily lower total, plasma testosterone than non-smokers.

5) Smokers will perform automatized tasks less well than non-smokers.

Study II

1) Smoking one standard cigarette will acutely reduce the frequency of EEG "driving" responses in both smokers and non-smokers.

9. Details of experimental design and procedures

Method

Study I

This is a comparative study in which the dependent variables listed below are compared in smokers versus non-smokers.

Subjects

Thirty, 21 to 30 year old, male volunteer smokers (at least one pack a day); and thirty, 21 to 30 year old, male volunteer non-smokers matched for educational level. All subjects will be in good health and not using any other drugs or medications.

Dependent Variables

Plasma MAO Activity

Five blood samples, each at the same hour of different days, will be drawn to determine the average plasma MAO activity of each subject. The method of Otsuka and Kobayashi (1964) will be

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

employed to assay plasma MAO activity.

EEG "Driving" Responses to Photic Stimulation

Two EEGs will be taken, one on the day of the fourth and one on the day of the fifth MAO blood sample to provide for adaptation to the EEG procedure. A third critical EEG will be taken on another day, after 3 hours of abstinence from smoking.

The EEGs will be obtained with a Grass model 5P5 electroencephalograph preamplifier. Bipolar occipital electrodes will be placed 2.5 cm to either side of the midline and between and parallel to the lines formed between the O_1-O_2 and P_3-P_4 derivations in the international 10-20 system of electrode placement. These electrodes are used to record the EEG responses to each of 18 photic stimulation trials. A Grass PS2 photic stimulator will be employed. The stimulation trials each last 10 seconds and are spaced 10-20 seconds apart, and are at 5, 10, 15, 20, 25 and 30 flickers/second. Each of these frequencies are administered at steps 2, 4 and 8 of the Grass intensity scale.

The EEG driving response is defined as the evocation of EEG waves at the fundamental or harmonic frequency of the photic stimulation for one full second, with no other EEG wave being visually detectable during that time.

The EEG driving response is scored as either present or absent for each stimulation trial. The EEGs will be independently scored by two scorers. Interscorer reliability in the past has been in the vicinity of .95.

Performance of Automatized Tasks

Automatization performances will be assessed by the method described in Klaiber, et al., (1967).

Anthropometric Indices of Testosterone Stimulation

Pubic hair development, and chest and biceps circumferences are each known to be affected by testosterone stimulation (Dorfman and Shipley); hence each will be employed as an index of endogenous testosterone stimulation.

The pubic hair ratings are made from photographs, with non-relevant portions of the picture eliminated, on a 5 point scale similar to that described by Tanner (1962).

The three anthropometric indices will be combined in the manner described by Klaiber et al., (1967).

Free and Total Plasma Testosterone and Serum LH and FSH

A blood sample for measurement of free and total plasma testosterone and for serum LH and FSH will be obtained on the day of the third plasma MAO blood sampling. The free and total testosterone will be measured by the method of Rosenfield (1971). The LH and FSH will be measured by the method of Odell (1967).

Study II

This is a manipulative study which examines the effects of smoking one cigarette on frequency of EEG "driving" responses. The cigarette would be smoked immediately after completion of the third, critical EEG trial of Study I. Another EEG trial will then be immediately obtained to allow comparison and with the preceding pre-smoking EEG trial.

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This procedure, i.e., two EEG trials within a short span of time, will be repeated on a later day without a cigarette interspersed between trials to control for the possibility that any main effect change in the second trial is due to some other factor, such as occipital neural satiation.

Subjects:

The same subjects as in Study I will be employed.

Cigarette:

A standard cigarette will be employed. Subjects will be requested to inhale. Non-smokers will be instructed, if necessary, how to inhale.

Future Work

The studies proposed here should logically be extended in various ways in the future. Measurement of blood testosterone metabolic clearance and production rates would be most important. These measures reflect the physiologic activity of testosterone more acurately than do blood testosterone levels.

The differences in adrenergic functioning believed to exist between male smokers and non-smokers should be investigated in females.

The psychophysiologic factors differentiating smokers and non-smokers may allow for prediction of future smoking behavior in young adolescents. This endeavor would require a longitudinal study across puberty. A longitudinal study of hormonal, psychological and physical changes occuring across puberty is currently being planned for our laboratory, and a smoking behavior study could easily be incorporated into this work.

Since prostaglandins appear to modulate norepinephrine release in the adrenergic nervous system, it would be important to assess differences in PG levels in smokers and non-smokers. The concentration of PGs is higher in seminal fluid than in any other type of tissue. Therefore, measurements should be made of seminal fluid PG E and F levels in smokers and non-smokers. We are currently carrying out a study on seminal fluid PG levels and the type of study discussed could be easily incorporated into this ongoing research.

10. Space and Facilities Available

The proposed research will be carried out in the laboratories of the Worcester Foundation for Experimental Biology and of Worcester State Hospital.

A fully equipped endocrine clinic is available at the Worcester Foundation. The assays for plasma MAO activity, total and free plasma testosterone, and serum LH and FSH are currently operative in the clinic laboratories.

The Psychology Department of Worcester State Hospital has a functioning research EEG laboratory with photic stimulation equipment. Cognitive testing facilities are also available in the Psychology Department.

All of the proposed procedures are currently being performed for other projects.

11. Additional Facilities required

None

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

References

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Hartman, B.K. & Udenfriend, S. The application immunological techniques to the study of enzymes regulating catecholamine synthesis and degradation. Pharmacol. Rev., 24, 311, 1972.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

References
(continued)

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Klaiber, E.L., Broverman, D.M., Vogel, W., Kobayashi, Y. & Hall, F. Effects of depo-testosterone administration upon plasma MAO activity in male patients. Submitted for publication, 1974.

Klaiber, E.L., Broverman, D.M., Vogel, W., Kobayashi, Y., & Moriarty, D. Effects of estrogen therapy on plasma MAO activity and EEG driving responses of depressed women. Amer. J. Psychiat., 128:12, 1492-1498, 1972.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

References
(continued)

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Youdim, M.B.H. Heterogeneity of rat brain and liver mitochondrial monoamine oxidase: subcellular fractionation. Biochem. Soc. Trans., 1, 1126, 1973.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Klaiber, Edward L., M.D., Principal Investigator	10%	$ 2,000
Cone, Frederick L., B.S., Senior Research Asst.	15%	2,000
Hawkins, Marion, R.N., Research Nurse	20%	2,000
Hall, Fernando, B.S., Research Assistant	25%	2,000
~~Technical~~ Consultants		
Vogel, William, Ph.D.	10%	2,000
Broverman, Donald, Ph.D.	10%	2,000
Sub-Total for A		12,000
B. Consumable supplies (by major categories)		
Glassward, chemicals and Sundry		1,800
Sub-Total for B		1,800
C. Other expenses (itemize)		
Subject costs		2,400
Fringe Benefit Charges on salaries at 18.5% (approx)		1,480
Sub-Total for C		3,880
Running Total of A + B + C		17,680
D. Permanent equipment (itemize)		
Sub-Total for D		none
E. Indirect costs (15% of A+B+C)		2,652
Total request		20,332

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O.K. LB

15. Estimated future requirements:

Depending on results obtained during the first year of study, an application for further support will be submitted in time for a January, 1976 continuation date. Further studies would be planned as outlined in the section on future work on page 9.

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Prostaglandins and other research for development of contraceptives	Agency for International Development Contract AID/csd-2837	$96,176	7/1/74 - 6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Mechanisms of Action of Gonadal Hormones in Depression	N.I.H. Research Grant MH 26145 - Approved But funding uncertain	$191,500	9/1/74 - 8/31/77
	N.I.H. Research Grant Unassigned - November 1974 Council	$183,300	1/1/75 - 12/31/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Edward L. Klaiber, M.D.

Signature Edward L Klaiber M.D. Date 7/30/74

Telephone 617 (Area Code) 757-7907 (Number) (Extension)

Checks payable to

:cester Foundation for Experimental Biology, Inc.

Mailing address for checks

222 Maple Avenue

Shrewsbury, MA 01545

Responsible officer of institution

Typed Name Federico Welsch, M.D., Ph.D.

Title Associate Director

Signature F. Welsch Date 7/30/74

Telephone 617 (Area Code) 842-8921 (Number) 414 (Extension)

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#929A LEETE

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 30, 1974

Grant application No. 929A

PHARMACOLOGY

To: The committee comprising Drs. Gardner, Jacobson, and Sommers

Subject: Edward Leete, B.Sc., Ph.D., D.Sc., University of Minnesota, Minneapolis
Continuation application No. 929A
"Synthesis and Biological Activity of Nicotine Analogs".

History

Grant No. 929 was awarded to Dr. Leete in the amount of $23,709 for the calendar year 1974, without assurance of continued support. Therefore, application No. 929A is in CTR parlance a "continuation", meaning that it competes on the same basis as a new proposal.

Request

Application No. 929A requests $24,155 plus one additional year.

Documents Submitted

Attached is application dated 7.16.74.

Reprints of the papers listed in item 3C of the application were provided, and will be forwarded to you if you wish.

F.W.N.

FWN:lb
Encs.

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#929A

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 29 1974

Application for Research Grant
(Use extra pages as needed)

Date 7.16.74

1. Principal Investigator (give title and degrees):

Edward Leete, Professor of Chemistry, B.Sc., Ph.D., D.Sc.

2. Institution & address:

University of Minnesota
Minneapolis
Minnesota, 55455.

3. Department(s) where research will be done or collaboration provided:

Department of Chemistry

4. Short title of study:

Synthesis and Biological Activity of Nicotine Analogs

5. Proposed starting date: 1.1.75 (This is a continuation of CTR grant No. 929)

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

Analogs of nicotine will be prepared for pharmacological testing. The biological tests will be carried out by Professor Von Euler, Karolinska Institute, Stockholm, and by Dr. Åke Pilotti of the Swedish Tobacco Company.

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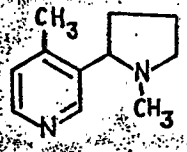
8. Brief statement of working hypothesis:

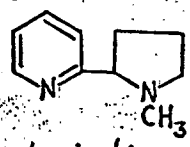
Our general plan is to prepare nicotine analogs for biological testing (see section 9. for the biological tests to be carried out) in the hope that we will learn something about the structural features required for "Nicotinic Activity" . Since there is considerable commercial interest in "synthetic tobacco" it is possible that some of these derivatives of nicotine which will be prepared will be more desirable components of the tobacco than nicotine itself. For example they could plausibly be added to a "synthetic tobacco" yielding a product with good smoking qualities but without some of the deleterious effects of nicotine.

9. Details of experimental design and procedures (append extra pages as necessary)

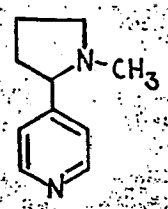
In our first progress report (covering the period 1.1.74 to 6.30.74) details of the synthesis of the following nicotine analogs are outlined . These compounds are being resolved into their optically active enantiomorphs and samples will be submitted to Dr. Von Euler



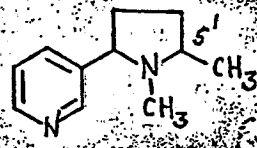
4-methylnicotine



α-nicotine



γ-nicotine

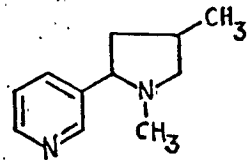


cis-5'-methylnicotine

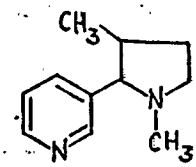
for the following tests :

1. Isolated rabbits jejunum
2. The guinea-pig ileum
3. The blood pressure of the cat
4. The isolated rectus abdominus muscle of the frog Rana temporaria

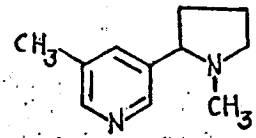
Dr. Åke Pilotti will test these and other analogs in a human cancer tissue culture which he has developed. Other analogs of nicotine which are to be made are illustrated below.



4'-methylnicotine



3'-methylnicotine



5-methylnicotine

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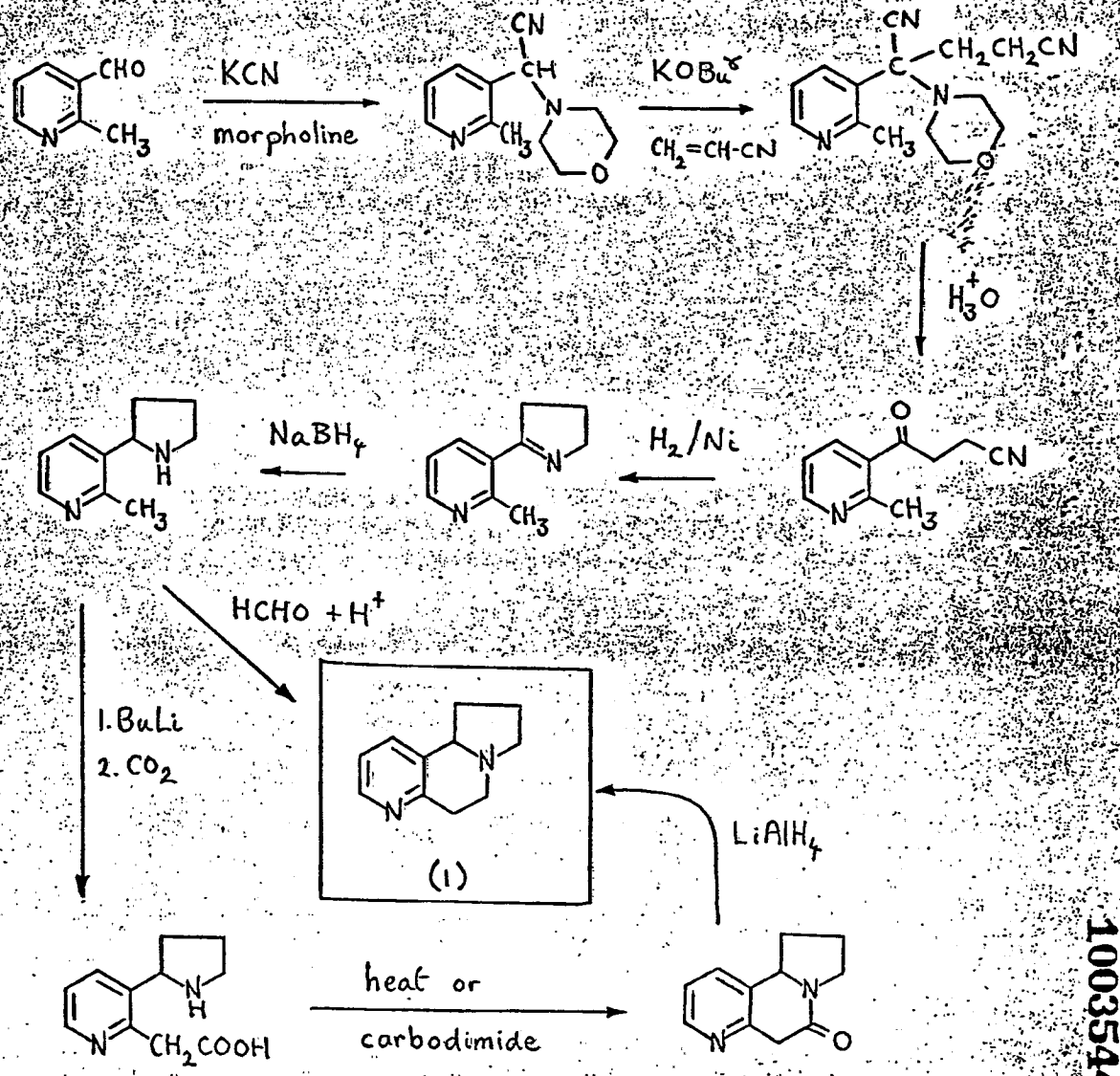
Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

The synthesis of these compounds will follow the general synthesis outlined in our original research proposal (Grant No. 929) . A compound of considerable interest is the tricyclic analog (1) . In this molecule the pyrrolidine ring will have a fixed conformation relative to the pyridine ring . The biological properties of this compound will be of great interest since it has been suggested that free rotation of the pyrrolidine ring relative to the pyridine ring is required for "Nicotinic Activity" . We plan to make this compound by the following route which we think has a reasonable chance of success. Previous attempts to make this compound have failed (F. Haglin, Acta Pharm. Suecica 4, 117 (1967)).



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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The chemistry department at the University of Minnesota has well equipped laboratories for chemical research. Most of my research group (currently 11 graduate students and one postdoctoral fellow) are housed in a new building (Kolthoff Hall) which was occupied in April 1970. I have a private research laboratory in which I take especial pride in working with my own hands for 30 to 40 hrs per week . My graduate students are in 4-man modules. The instruments available to my group are the following: Chromatronix Series 3500 high pressure liquid chromatograph, 2 Gilson fraction collectors, an LKB fraction collector, a Varian XL-100 nmr spectrometer (with Fourier transform C-13), and AEI-MS-30 high and low resolution mass spectrometer, a Cary 11 uv spectrophotometer, a Perkin Elmer ir spectrophotometer,a Cary 60 ord spectrophotometer, a Nuclear Chicago Mark II liquid scintillation counter. A greenhouse is available on the roof of the chemistry building where tobacco and other plants are grown. Rooms are also available for the extraction of plants, for hydrogenations, and for the culture of micro-organisms.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Edward Leete (principal investigator) Philip Hoekstra (graduate student)
Tony Catka (graduate student)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

list appended

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

Item 12 (continued)

Biographical sketches of the professional personnel

Edward Leete (Principal Investigator)

REDACTED

REDACTED

REDACTED

Philip Hoekstra (3 rd. year graduate student)

REDACTED

REDACTED

Tony Catka (1 st. year graduate student)

REDACTED

REDACTED

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Item 13

Recent Publications (reprints are attached at the end of this application)

1. E. Leete and Yu-Ying Liu , Metabolism of [2-^{3}H]- and [6-^{3}H]-nicotinic acid in intact Nicotiana tabacum plants. Phytochemistry, 12,593 (1973).

2. E. Leete, Biosynthesis and Metabolism of the Tobacco Alkaloids, Proceedings of the First Philip Morris Science Symposium, Nicholas J. Fina, editor, 1973, p.92.

3. E. Leete and G. B. Bodem, Biosynthesis of Shihunine, an Alkaloid of the Orchid Dendrobium pierardii, J. C. S. Chem. Commun., 522 (1973).

4. E. Leete, Biosynthesis of Alkaloids, Chapt. 4 in Biosynthesis 2 , ed. T. A. Geissman, A Specialist Periodical Report of the Chemical Society, London, 1973, p.106.

5. E. Leete, G. E. Davis, C. R. Hutchinson, K. W. Woo, and M. R. Chedekel, Biosynthesis of Azetidine-2-carboxylic Acid in Convallaria majalis, Phytochemistry, 13, 427 (1974).

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Edward Leete (one month summer salary)		REDACTED
Philip Hoekstra (research assistant)		
Tony Catka (research assistant)		
Fringe benefits on the above salaries (calculated at 17.5%)		
Technical:		
Sub-Total for A		R
B. Consumable supplies (by major categories)		
Chemicals (organic compounds, solvents etc.)		3500
Glassware		1500
Sub-Total for B		5000
C. Other expenses (itemize):		
Analytical services (mass spectra, nmr, CHN)		600
Page charges for journal articles		300
Travel to one domestic scientific meeting		300
Sub-Total for C		1200
Running Total of A + B + C		21005
D. Permanent equipment (itemize):		
none		
Sub-Total for D		0
E. Indirect costs (15% of A+B+C)		3150
Total request		24155

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	16000	5000	1200	0	3300	25530
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Synthesis and Biological activity of nicotine analogs	Council for Tobacco Research No. 929	$23709	01/01/74 to 12/31/74
Metabolism of Natural Products of Medicinal Interest	NIH-GM-13246-17 (the terminal year of a 5 year grant period	$ 46810	01/01/74 to 12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Metabolism of Natural Products of Medicinal Interest	NIH-GM-13246-18 (Funds are tight in this branch of the NIH and it is dubious that this full amount will be granted)	$ 63615	01/01/75 to 12/31/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Edward Leete

Signature Edward Leete Date 7.16.74

Telephone 612 (Area Code) 373 (Number) 2380 (Extension)

Checks payable to

University of Minnesota

Mailing address for checks

302 Morrill Hall

University of Minnesota

Minneapolis, MN, 55455.

Responsible officer of institution

Typed Name Clinton T. Johnson

Title Asst. Vice President, Business Administration

Signature X C. T. Johnson Date

Telephone 612 (Area Code) 376 (Number) 7614 (Extension)

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#955 WEISS

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

February 6, 1974

Grant application No. 955

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner and Liebow

Subject: George B. Weiss, Ph.D., Univ. of Texas Southwestern Medical School, Dallas
New application No. 955
"Agents which Alter the Transport, Storage and Distribution of Nicotine"

History

This proposal was case #247 and formal application was encouraged. Until December 31, 1974, Dr. Weiss's research will be supported by the AMA Educational and Research Foundation.

Request

Application No. 955 requests $25,287 plus two additional years.

A starting date of January 1, 1975 is requested, to "mesh" with support from AMA-ERF.

Document Submitted

Attached is application dated January 22, 1974 (7 pages).

F.W.N.

FWN:gh

Encls.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

CTR.

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022

RECEIVED
SEP 24 1974
H. WAKEHAM

September 19, 1974

Dr. Helmut R. R. Wakeham
Vice President
Corporate Research & Development
Philip Morris Inc.
P. O. Box 26583
Richmond, Virginia 23261

Re: Our grant application No. 955
George B. Weiss, Ph.D.

Dear Wake:

Thank you very much for your letter of September 17, providing cogent comments on the proposal we have received from Dr. Weiss.

Copies are being immediately distributed to all concerned with action on this application.

Sincerely,

Fred

FWN:lb

Frederic W. Nordsiek, Ph.D.
Associate Research Director

1003544541

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

cc: T. S. Osdene
R. Fagan

September 17, 1974

Dr. Frederic W. Nordsiek
Associate Research Director
The Council for Tobacco Research-U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Fred:

Re: Our grant application No. 955
George B. Weiss, Ph.D.

With reference to your letter of September 5, 1974, the following are my comments on Dr. Weiss' application:

1) If one takes the title literally, the method described will not reveal anything about the "...transport, storage and distribution" of nicotine let alone their alteration. Since he proposes to use tissue slices rather than whole animals or cells, Weiss cannot tell anything about transport or about storage. Perhaps he will be able to make statements about distribution.

2) Although I am not particularly knowledgeable in this field, I agree with the comments made by Dr. Pohorecky. She points out the many places in which Dr. Weiss is vague. She feels, as I do, that what Dr. Weiss proposes to do will not answer the questions implied in the title.

Sincerely,

H. Wakeham, Vice President
Research and Development

Encl.

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Dr. Ray Fagan } Pls
Dr. T. S. Osdene } handle
You may sign my name to your comments but please keep me informed
Hwake

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022

September 5, 1974

App. 955
in SAB books
Gave to Fagan
9/17

Dr. Helmut R. R. Wakeham
Vice President
Corporate Research & Development
Philip Morris Inc.
P. O. Box 26583
Richmond, Virginia 23261

Re: Our grant application No. 955
George B. Weiss, Ph.D.

Dear Wake:

In the Scientific Advisory Board agenda books sent to you last week, you will find the above cited application. The staff here have expressed the hope that you might be prepared to comment at the meeting on Dr. Weiss' application.

If you care to send a written commentary in advance, so much the better. In that event, please just mail the original here, and we would distribute appropriate copies.

To give you full background, enclosed is a kit that was sent to the SAB committee, but excluded from the books. These documents include updating by the applicant, evaluations by two outside consultants, and evaluations by three SAB members.

Thanks and best wishes.

Sincerely,

Fred

Frederic W. Nordsiek
Associate Research Director

FWN:lb
Encs.

cc: Dr. William U. Gardner
Dr. Robert C. Hockett
Dr. Sheldon C. Sommers

10035445

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

June 21, 1974

Grant Application No. 955

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner and Liebow

Subject: George B. Weiss, Ph.D., University of Texas Southwestern Medical School, Dallas
New application No. 955
"Agents which Alter the Transport, Storage and Distribution of Nicotine"

You are seeing this proposal for the second time. It was sent to you on February 6, 1974, but was withheld from the March 1974 agenda because funding was requested only as of January 1, 1975. (The interim has been well spent in obtaining evaluations).

To give you full data we enclose the following:

1. FWN to committee, February 6, 1974, with application.

2. Dr. Weiss to FWN, June 18, 1974, updating proposal.

3. Evaluations:

   a. Dr. Essman, February 25, 1974
   b. Dr. Pohorecky, March 1, 1974
   c. Dr. Bing (undated)
   d. Dr. Liebow (undated)
   e. Dr. Gardner, February 19, 1974 (Re last sentence: Dr. Miller was approached for an evaluation but suggested Dr. Pohorecky in his stead.)

F.W.N.

FWN:wg
Encls.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

February 6, 1974

Grant application No. 955

PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner and Liebow

Subject: George B. Weiss, Ph.D., Univ. of Texas Southwestern Medical School, Dallas
New application No. 955
"Agents which Alter the Transport, Storage and Distribution of Nicotine"

History

This proposal was case #247 and formal application was encouraged. Until December 31, 1974, Dr. Weiss's research will be supported by the AMA Educational and Research Foundation.

Request

Application No. 955 requests $25,287 plus two additional years.

A starting date of January 1, 1975 is requested, to "mesh" with support from AMA-ERF.

Document Submitted

Attached is application dated January 22, 1974 (7 pages).

FWN

F.W.N.

FWN:gh

Encls.

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THE UNIVERSITY OF TEXAS
HEALTH SCIENCE CENTER AT DALLAS

DEPARTMENT OF PHARMACOLOGY

SOUTHWESTERN MEDICAL SCHOOL
GRADUATE SCHOOL OF BIOMEDICAL SCIENCES
SCHOOL OF ALLIED HEALTH SCIENCES

June 18, 1974

Frederic W. Nordsiek, Ph.D.
Associate Research Director
The Council for Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Re: Application No. 955

Dear Dr. Nordsiek:

This letter is in response to your request of February 27, 1974, to update application no. 955 (Agents which Alter the Transport, Storage and Distribution of Nicotine) prior to consideration for funding in October, 1974.

Since the projected research had a proposed starting date of January 1, 1975, we have made substantial progress over the past few months on only one of the specific experimental approaches [specific aim "a", part (1)]. The major portion of this data has now been obtained and it is anticipated that all work on this problem will be completed by early fall. Though it is difficult to summarize all of these findings at this time, it is clear that our techniques can distinguish between those agents specifically competing with nicotine at intracellular binding sites and others with less specific mechanisms of action.

Changes in references (page 2c) are as follows:

8. MS in preparation to MS submitted (Neuropharmacology).
19. unpublished data to Neuropharmacology, In Press.

If there is any additional information or materials which I can furnish the Scientific Advisory Board to assist in evaluation of this application, please let me know. Thank you for your assistance.

Sincerely yours,

George B. Weiss, Ph.D.
Associate Professor of Pharmacology

GBW/lh

5323 HARRY HINES BLVD. DALLAS, TEXAS 75235 (214) 631-3220

100354546

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#955

QUEENS COLLEGE

of THE CITY UNIVERSITY OF NEW YORK

FLUSHING • NEW YORK 11367

February 25, 1974

Dr. Frederick W. Nordsiek
Associate Scientific Director
The Council for Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York City, New York 10022

Dear Dr. Nordsiek:

I have read and reviewed the grant application that you sent to me written by George B. Weiss and will try to comment briefly on some of the points in this proposal which might bear upon its consideration by the council.

In general, I found this to be a very interesting proposal, but one which is extremely broad in scope rather than specific in intent. I make this comment primarily based upon the investigation that the working hypothesis, while based upon the premise that intracellular storage, binding and movement of biogenic amines is altered by nicotine and that these alterations presumably are site-specific for the mammalian central nervous system, there is no indication, at least in the reference cited on Pg. 2C, that this is really supported, unless it has been dealt with in the one paper which is listed as a manuscript in preparation.

The use of washout experiments may be generally useful, but again the ultimate determinant of the effectiveness of such methodology would seem to rely upon gross tissue assay procedures utilizing labelled amines. To accomplish the goals set forth for the working hypothesis underlying this series of experiments, it would seem that more than just gross tissue analysis, regardless of methodology, would be a requisite; namely the subcellular distribution of such amines would appear to be a necessity and this would similarly apply, particularly in those experiments in which C^{-14} nicotine uptake was being measured. Mention was made of the fact that C^{-14} nicotine uptake can be inhibited by several compounds related to the cholinergic system; and yet there is no compound in the proposal to examine the relationship between nicotine uptake of acetylcholine or other choline esters. Mention was made of intracellular binding sites for nicotine and possible mechanisms of complete inhibition exerted by those agents which inhibited its uptake. However, no effort was made to either investigate such binding sites, nor is there any indication that the interaction will be further considered.

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The use of bioassays, as proposed on Pg. 2B, Section 3, which does not represent a particularly new area of investigation for the effects of nicotine, unless there is some further underlying rationale, is not specified here, for relating regional brain uptake with peripheral muscle response.

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

I cannot, offhand, conceive of any such rationale.

It would be of considerable usefullness to have some basic information upon the dose-response relationship between nicotine and biogenic amine accumulation by the central nervous system tissue. It is quite conceivable that non-physiologic doses of the alkaloid will indeed modify amine distribution in vitro, which can be demonstrated with veratrines, vinca, and other groups of rather distant chemical relationships to nicotine. It would seem that the experiments proposed in Sections 1 and 2 on Pg. 2B would rely upon such information, but have not specifically proposed its consideration, although there is some mention in this portion of the proposal of intracellular sites, which seems a rather ambitious undertaking to examine "binding sites within the cell membrane", and I would be most curious to know how these would be undertaken. It is, furthermore, not clear whether such investigation would be strictly in vitro studies, using isolated, incubated tissue slices, or whether the uptake tissue studies would be carried out in vivo and the local studies examined in vitro.

My general impression of this proposal is that it is a rather ambitious undertaking which will ultimately rely upon some highly sophisticated methodology in cellular biology. Much of this methodology has not been detailed in this proposal and some of the experiments would actually be based upon such procedures which I am not aware of having yet been developed or published. My feeling is that if this investigator has already proved his competence beyond investigating drug effects upon gross tissue distribution of labelled amines, then the experiments proposed might be worthwhile investigating. If, however, the entire proposal rests on the assumption that the relationship between uptake, distribution and disposition of nicotine and its interactions with other amines or alkaloids and remains to be established by this series of studies, then it would still appear to me to be highly ambitious as an undertaking.

I hope that these comments are useful to you in the evaluation of this proposal and if there are any more specific areas within which you would like me to report, I would be happy to do so.

Sincerely yours,

Walter B. Essman, M.D., Ph.D.
Professor

WBE/nm

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#955

THE ROCKEFELLER UNIVERSITY

NEW YORK, N.Y. 10021

March 1, 1974

Frederic W. Nordsiek, Ph.D.
Associate Scientific Director
The Council for Tobacco Research-USA, Inc.
110 East 59th Street
New York NY 10022

Dear Dr. Nordsiek:

Re: Project on Agents which Alter the Transport, Storage and Distribution of Nicotine

George B. Weiss, Principal Investigator

Although the hypothesis and the specific aims of this proposal are sound, interesting, and worthwhile to investigate, I believe that the rest of the proposal is very poor. The whole approach is open to question, and it is difficult to see how some of the experiments relate to the specific aims. The insufficient description of the experiments and methods do not give the reader a clear picture of what the investigators really are planning to do in order to examine some of their research aims. Therefore I would judge this application completely inadequate.

Some Specific Points:

I. General Procedure

Why are the animals going to be lightly anesthetized before being decapitated? Adding this variable is unnecessary, and may prove tricky. Did, or will, the investigators check what effects even a brief ether exposure might have on the parameters to be subsequently measured? Ether is a well-known releaser of glucocorticoids from the adrenal cortex. Glucocorticoids have been shown to alter the uptake of norepinephrine by brain slices (Maas and Mednieks, Science, 1971, 171, 178-179). We and others have evidence that a very brief exposure to ether alters the uptake and turnover of ^{3}H-norepinephrine.

The description of the methods and procedures is very inadequate. No time periods are specified for incubations and treatments. Are the tissue slices going to be pre-equilibrated? It is difficult to assess their procedures with brain slices, and involving ^{3}H-norepinephrine, since reference 8 is not yet in press. Is the uptake of ^{3}H-norepinephrine going to be carried out in the presence of an MAO inhibitor? If not, a considerable portion of the labeled norepinephrine will be metabolized during the incubation period. In the part describing the washout procedure, it is mentioned that slices will be ashed "as in the just described uptake experiments." However, ashing of slices was not mentioned under the uptake procedures.

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It is not clear whether all the drugs to be tested are to be added in vitro only or whether they will also be injected into animals.

II. Specific aim "a"

1) The subheading states that effects of various agents on transport, distribution, and storage of nicotine will be examined. However, no experiments are described that will test these factors. For instance, how does one study the transport and distribution of nicotine in brain slices? Does transport mean uptake?

2) None of the experiments mentioned under methods or experimental approaches are directed toward "delineating the sites" of ^{14}C-nicotine uptake. The subheading states "examination of nicotine analogues and antagonists on ^{14}C-nicotine distribution." Do they mean regional distribution in the brain, or do they mean subcellular distribution within neurons? This is completely unclear, and the experiments that are mentioned do not allow one to tak a guess.

3) How do the investigators propose to distinguish between effects and movements of nicotine in central and peripheral systems? No indication of this is given.

III. Specific aim "b"

1) The investigators do not provide any background information on the effects of nicotine on the distribution of biogenic amines. Their reference 19, which would provide some of this information, is not published. Previous work by other investigators has shown that chronic treatment with nicotine results in an increased uptake and turnover of norepinephrine (Bhagat, Brit. J. Pharmacol. 1970, 38, 86, for example). Also, Balfour showed that nicotine added in vitro increased the initial uptake of ^{14}C-norepinephrine but decreased the retention of this amine, especially in the hypothalamus, while the uptake of ^{14}C-serotonin was decreased in both hypothalamus and hippocampus (Eur. J. Pharmacol. 1973, 23, 19).

How do the investigators propose to study the binding of ^{3}H-amines to sites "within the cell membrane"? As far as I am aware, no such procedures are yet available for ^{3}H-norepinephrine in brain, and, if they plan to test a new procedure, they do not state so.

One other point: To check if the uptake of ^{14}C-nicotine occurs in noradrenergic terminals, the investigators should use slices from animals treated with 6-hydroxydopamine, which preferentially destroys noradrenergic and dopaminergic nerve terminals. This was done, for example, by Westfall and Brasted (J. Pharmacol. Exp. Ther. to check nicotine uptake into noradrenergic nerve terminals in the heart.

In conclusion, then, the experiments presented in this proposal will

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not enable the investigators to obtain the information that their specific aims call for.

I hope you will find these comments helpful.

Sincerely yours,

Larissa A. Pohorecky, Ph.D.

LAP:emg

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THE COUNCIL FOR TOBACCO RESEARCH--U.S.A., INC.

New Application No. 955

George B. Weiss, Ph.D., University of Texas, Dallas

file 955 (Recirculate Fall 1979)

This is on "Agents which alter the transport, storage and distribution of nicotine". In addition to that, he is interested in delineating the sequence of events important for the transfer and/or release of biogenic amines by nicotine.

This is a well-plowed field. He has to be extremely original to make an impact. What is at stake is an investigation into the different ways in which nicotine is stored, concentrated and accumulated in brain tissue, studies which have been primarily performed in Sweden. The technique will consist of brain sections and ^{14}C-labelled compounds such as serotonin, ^{14}C-nicotine, etc.

The experimental approach is to use pharmacological agents on nicotine efflux on the examination of the effects of nicotine, on its distribution and its antagonists and anolox, on the interaction between nicotine and other amines.

I do not think that this is a very original work. It is not bad, and it is not very good. 3-4 on scientific merit, and 3-4 on relevance.

R. J. Bing, M.D.

RJB:cc

cc: Drs. Gardner, Liebow and Nordsiek

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SUBJECT: Application #955
George B. Weiss, Ph.D., University of Texas Southwestern Medical School, Dallas
"Agents which alter the transport, storage and distribution of nicotine".

This study is directed toward investigation of agents that inhibit the transport-mediated uptake of nicotine or its cellular binding, and the events affecting the "translocation" or release of biogenic amines by nicotine. The method used is determination of the concentration of appropriate radio-labeled substances in brain slices exposed to concentrations of the agents to be investigated. Stress will be placed on inhibitors such as procaine and mecamylamine, and the effects of nicotine analogs and antagonists. Comparison of effects in the central nervous system with those in the periphery, such as voluntary or smooth muscle preparations, will be made. A second major effort will be on the interaction between nicotine and biogenic amines, where existing evidence suggests that nicotine alters the distribution of these substances.

CRITIQUE:

Drs. Weiss and Goodman have already had extensive experience in the use of the proposed method. This has the advantage of providing quantifiable results, but the test system is extracorporeal and thus artificial. The technique is capable of providing only very roughly focused results in terms of localization that might be achievable by the autoradiographic and histochemical methods now readily available. While the budget is modest, this work does not give promise of producing any specially knowledgeable results or concepts.

A.A. Liebow, M.D.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 19, 1974

MEMORANDUM

TO: Drs. Bing, Hockett, Liebow, Nordsiek and Sommers

FROM: W. U. Gardner

SUBJECT: Application 955.
George B. Weiss, Ph.D., University of Texas Southwestern Medical School, Dallas.
"Agents which Alter the Transport, Storage and Distribution of Nicotine."

This is a request for continuing support of a former AMA-ERF grantee ($52,358 direct costs, 2/1/73-1/31/75). They have reported some work on nicotine on rat rectus muscle and have one paper on 5HTP efflux by nicotine in 1973. They have a big NIH application in for a mechanism of drug action project.

They propose an in vitro study using rat brain slices from several areas. Standard methods will be used for the uptake and the washout of ^{14}C-labeled nicotine, seratonin, norepinephrine, dopamine and nicotine uptake in the presence of some blockers such as decamethonium, curare and nicotine metabolites.

This seems to be a very self-limiting method of making advances at this time. Autoradiography would certainly give much more localization, and because of the heterogeneity of brain tissue, almost as much quantitation. Frozen section techniques are available so the even water-soluble substances can be localized. It will probably do nicotine localization shortly by immunofluorescent methods, at least of slices. Histochemical means and vescicle counts can be used to approximate some biogenic amines. ACh is not mentioned.

I suggest that Essman and Niel Miller might be ad hoc consultants.

W.U.G.

WUG:ek

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GL

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

RECEIVED JAN 28 1974

Date: January 22,1974

1. Principal Investigator (give title and degrees):

George B. Weiss, Ph.D.
Associate Professor of Pharmacology

2. Institution & address:

University of Texas Southwestern Medical School
5323 Harry Hines Boulevard
Dallas, Texas 75235

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Agents which Alter the Transport, Storage and Distribution of Nicotine

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

The specific aims for the initial period of requested support are:

a. To examine, in a comparative and systematic manner, the precise mechanisms by which various pharmacological agents alter the transport, distribution or storage of nicotine. Primary attention will be given to use of agents or procedures which inhibit the transport-mediated uptake of nicotine or the intracellular binding of nicotine.

b. To delineate the sequence of events important for the translocation and/or release of biogenic amines by nicotine. Characterization of relevant parameters will be attempted by use of selected agents which (1) directly antagonize the action of nicotine, (2) alter ionic concentrations important for coupling the action of nicotine to that of amine release, or (3) specifically affect the resultant movement of biogenic amines.

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8. Brief statement of working hypothesis:

The working hypothesis for this project is that important actions of nicotine in the central nervous system are based upon specific alterations in intracellular storage, binding and movements of biogenic amines. These effects depend at least partly upon the different ways in which nicotine is accumulated and concentrated in brain tissue. More specifically, there appears to be not only a passive distribution of nicotine in accord with cellular pH gradients but also an active energy-dependent uptake and retention of nicotine in various brain areas. The interactions between nicotine accumulated in this manner and endogenous stores of biogenic amines present in some brain areas provide a logical focal point for examination of the mechanisms by which central effects of nicotine are induced. In an analogous manner, characterization of the specificity of transport system responsible for a portion of the nicotine accumulation should provide information about the type or types of agents which either utilize or inhibit this process.

9. Details of experimental design and procedures (append extra pages as necessary)

a. General Procedures

Brain sections are obtained from male Wistar rats weighing 180-250 g. All animals are decapitated while under light ether anesthesia, brains rapidly removed, and brain areas prepared for slicing by dissection on filter paper which has been moistened with bathing solution and placed on ice. The general dissection procedure has been described (1). The specific procedure to be used involves preparation of two slices from each of 3 different brain areas. First, one slice is cut from the lateral surface of the cortex of each hemisphere. Second, the corpus callosum is removed, the two striatum sections dissected out, and each striatal portion is sliced in half with the two halves considered as a single slice. Third, the brain is turned over and the hypothalamus isolated and divided in half at the midline. Each half is sliced in two and the two parts are treated as a slice. Slices (approximately 0.5 mm thick) are prepared with a longitudinal vibrating monofilament nylon thread (2) and incubated at 37°C in a bicarbonate-buffered salt solution at pH 7.3.

In experiments measuring uptake and washout of ^{14}C-labeled compounds, the procedures employed would be similar to those described for ^{14}C-serotonin in smooth muscle (3,4), ^{14}C-nicotine in striated muscle (5-7) and brain area slices (8), and ^{14}C-serotonin in brain area slices (9). For uptake measurements, slices are placed in tubes containing aerating bathing solution plus radioisotope. After the desired incubation interval, slices are removed from the radioactive solution, dipped rapidly into two consecutive tubes containing nonradioactive solutions similar to the incubation solution (to remove radioactive bathing solution adhering to the surface of the slices), and drained by gently rolling on a glass surface until essentially all adherent solution is removed. Then slices are weighed on a Federal-Pacific torsion balance (sens. 0.05 mg) and placed in test tubes; the ^{14}C-labeled compound is removed by dialysis or extraction. Aliquots from the resultant solution are placed into appropriately prepared planchets, dried and counted in a Nuclear-Chicago proportional gas flow counter. Uptake can be expressed as a tissue space (ml/g) or as tissue uptake (micromoles per gram of tissue).

Washout experiments are similar to uptake experiments in most details. A special holder is employed to facilitate serial transfer of freely floating slices (10). After incubation and dipping, the slices are transferred through a series of test tubes, each of which contains a known volume of aerated nonradioactive medium (11). Upon completion of the washout, each slice is drained by gentle rolling, weighed, and ashed as in the uptake experiments. Curves delineating (a) the decline of tissue radioisotope

(continued on page 2a)

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with time, expressing this as a percentage of the initial isotope content (desaturation curve), and (b) the percentage of the remaining isotope washed out of the tissue per minute of each collection interval (rate coefficient curve) were constructed. Both of these plots have been used to describe loss and alterations in loss of radioisotopes (12,13).

Assay procedures employed for quantitative analysis of ^{14}C-serotonin and its metabolites are described in previous studies with isolated smooth muscle (3,4) and brain area slices (9). Quantitative detection of catecholamines (norepinephrine, dopamine, and metabolites) will be accomplished by isotopic (e.g. 14-16) techniques; tissues would be exposed to either ^{14}C-labeled norepinephrine or ^{14}C-labeled dopamine. Separation of catecholamines and their metabolites will be accomplished by a modified method based on that of Neff and Costa (17). Brain area slices would be homogenized in 4 ml of 0.4N perchloric acid and, after centrifugation, an aliquot of the clear supernatant will be taken and total radioactivity determined. To determine the relative proportions of ^{14}C-labeled catecholamines and their metabolites present, the remaining supernatant will be placed in a glass-stoppered centrifuge tube containing prepared alumina (18) and the pH of the mixture will be adjusted to pH 8.2-8.4 with 0.5 M tris buffer. After centrifugation and separation, catechols will be eluted from the alumina with 0.4N acetic acid.

b. Specific Experimental Approaches

An outline of the initial areas of investigation indicated by the specific aims (#7) can be provided. Most of the techniques to be employed have been or are being carried out in this laboratory. Consequently, serious technical difficulties are not anticipated. The initial experiments are designed to reveal the most relevant and accessible relationships in as efficient a manner as possible.

Specific aim "a" experimental approaches:

(1) Characterization of effects of appropriate pharmacological agents on transport, distribution and storage of nicotine in slices from different rat brain areas. Initially, effects of various agents on ^{14}C-nicotine efflux and uptake will be ascertained. Attention will be focused on the mechanisms of action of agents known to inhibit uptake of ^{14}C-nicotine. Preliminary experiments show that ^{14}C-nicotine uptake was markedly inhibited by procaine, mecamylamine, physostigmine (but not acetylcholine) and lobeline, and increased by high (depolarizing) potassium ion concentrations. Further experiments with these agents are planned to determine whether some (physostigmine?, lobeline?) may specifically compete with nicotine for intracellular binding sites whereas others (procaine?, high potassium?) may act by altering cell polarization and permeability.

(2) Examination of effects of nicotine analogues and antagonists on ^{14}C-nicotine distribution and accumulation. Following delineation of sites at which ^{14}C-nicotine is taken up and accumulated, it would be practical to examine the actions of nicotine-related agents in the same manner. This group of agents includes nornicotine (a nicotine precursor), cotinine (a nicotine metabolite), nicotine analogues (e.g. nicotine monomethiodide) and nicotine antagonists (e.g. DMAE). It is anticipated that newer nicotine antagonists will be developed in the near future and will be made available to us. For example, Dr. John Long (Univ. of Iowa) has recently sent

(continued on page 2b)

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us a supply of a new derivative of DMAE (TP-DMAE) which has no sympathomimetic activity. It would be of interest to examine the effects of this new junctionally-active agent on nicotine-induced effects and distribution in brain area slices.

(3) Development of isolated central and peripheral preparations for measuring and comparing nicotine antagonist activity. The studies on effects of pharmacological agents and nicotine-related compounds on ^{14}C-nicotine in brain area slices would be compared with relevant analogous interactions in isolated peripheral systems in which junctional or ganglionic actions of nicotine can be readily examined (e.g. frog rectus abdominis muscle, guinea pig ileal preparation). It is necessary to distinguish between effects and movements of nicotine important in both central and peripheral systems and those actions of nicotine which are present only in central nervous system tissue. As an example, do the standard nicotine junctional blocking agents (curare, decamethonium) affect brain area distribution of nicotine? Conversely, is atropine a more effective central antagonist of nicotine? Resolving these interrelationships would be necessary to dissociate antagonist activities at different types of nicotinic sites or stores.

Specific aim "b" experimental approaches:

(1) Interactions between nicotine and biogenic amines. Previous studies on the action of nicotine on ^{14}C-serotonin distribution (9) and related preliminary data with ^{14}C-norepinephrine and ^{14}C-dopamine (19) indicate that nicotine has specific effects on the distribution of these biogenic amines in striatum or hypothalamus slices. The critical question is whether these effects of nicotine represent specific interactions between nicotine and a biogenic amine at intracellular sites or, alternatively, represent a sequential reaction in which one or more intermediate steps serve to couple the specific action of nicotine with a translocation of cellular amine. Two approaches will be used initially to clarify this point. First, we will further examine specific effects of nicotine on the cellular distribution and accumulation of appropriate ^{14}C-labeled amines to ascertain whether the amine stores affected are primarily at binding sites within the cell membrane, within intracellular depots (granules?), or at nonspecific intracellular locations. Secondly, we would examine the effects of biogenic amines upon ^{14}C-nicotine accumulation. Presumably, if nicotine and amines interact at specific cellular binding sites, these amines should have effects on ^{14}C-nicotine similar to those which nicotine has on ^{14}C-labeled amines.

(2) Delineation of mechanisms of nicotine-amine interactions. Subsequent to determination of nicotine-amine interactions, we would examine the influence of a variety of parameters which may affect differing aspects of these relationships. These would include duration of exposure (transient or maintained effect?), effects of temperature and metabolic inhibitors (nonspecific energy-dependence of effects?), and actions of ouabain and of variation of ionic concentrations (specific effects on energy-dependent transport?).

(continued on page 2c)

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

References

1. Hertz, Schousboe and Weiss, Acta Physiol. Scand. 79: 506, 1970.
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16. Whitby, Axelrod and Weil-Malherbe, J. Pharmacol. Exp. Ther. 132: 193, 1961.
17. Neff and Costa, Life Sci. 5: 951, 1966.
18. Crout, Standard Methods of Clinical Chemistry, Vol. 3, p.62. Academic Press, New York, 1961.
19. Goodman, unpublished data.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

a. Space available: Fully equipped new laboratory facilities suitable for radioisotope and related work.

b. Facilities available:

(1) Two automated Nuclear-Chicago planchet counting systems (one low background).
(2) An Olivetti Programma 101 and several electronic calculators.
(3) Cahn electrobalance, torsion and analytical balances, pH meters, heaters, muffle furnaces, constant temperature baths, recorders.
(4) Custom designed slicer and related equipment for brain slice preparation.
(5) Grass model 7 polygraph with Model 7P1 Low-Level D.C. Preamplifiers and FT.03 strain gauge transducers.

Also in the department and readily available are:

(6) Beckman Model 979 Atomic Absorption-Spectrophotometer system.
(7) A variety of centrifuges including a Beckman L2-65B Ultracentrifuge.
(8) Analytical equipment and facilities for chemical procedures required for isolation and analysis of drugs and their metabolites.
(9) Equipped small animal room.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See page 6

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See page 7

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
a. G. B. Weiss, Ph.D.	40	none
b. F. R. Goodman, Ph.D.	35	6125.
Technical		
c. Research Technician II (G. Atkins or to be recruited)	100	8724.
d. Secretary - Linda High	25	1725.
Fringes for b, c and d		1217.
Sub-Total for A		17,791.
B. Consumable supplies (by major categories)		
a. Chemicals		350.
b. Glassware		200.
c. Animals (mainly rats, rabbits)		850.
d. Isotope and related supplies		600.
e. Publications, office supplies and related expenses		700.
Sub-Total for B		2700.
C. Other expenses (itemize)		
a. Maintenance - Nuclear Chicago Counter		600.
b. Travel (FASEB and/or ASPET meetings)		550.
Sub-Total for C		1150.
Running Total of A + B + C		21,641.
D. Permanent equipment (itemize)		
a. Incubation baths, heaters, tissue holders and related minor equipment		400.
Sub-Total for D		400.
E. Indirect costs (15% of A+B+C)		3246.
Total request		25,287.

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	18,858	2700	1150	400	3406	26,514
Year 3	19,989	2700	1150	400	3576	27,815

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
a. Interactions of Nicotine with Cellular Transport Systems on Muscle and Nerve	AMA-ERF	$52,358 direct cost	2/1/73-1/31/75
b. Mechanisms of Drug Action in Vascular Smooth Muscle	NIH HL-14775	$65,835 direct cost	9/1/71-8/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
c. Mechanisms of Drug Action in Vascular Smooth Muscle	NIH HL-14775	$166,275 direct cost	9/1/74-8/31/79

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name George B. Weiss

Signature George B. Weiss Date 1-22-74

Telephone 214 (Area Code) 631-3220 (Number) 2268 (Extension)

Checks payable to

Univ. of Texas Southwestern Medical School

Mailing address for checks

Mr. Julius E. Weeks
Univ. of Texas Southwestern Medical School
5323 Harry Hines Boulevard
Dallas, Texas 75235

Responsible officer of institution

Typed Name F. J. Bonte, M.D.

Title Dean, Univ. of Texas Southwestern Medical School

Signature F. J. Bonte Date 1-22-74

Telephone 214 (Area Code) 631-3220 (Number) 601 (Extension)

100354562

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1003544563

MISCELLANEOUS

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

1003544564

#999 HAVLICEK

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

July 30, 1974

Grant application No. 999

MISCELLANEOUS

To: The committee comprising Drs. Bing, Jacobson, and Sommers

Subject: Victor Havlicek, M.D., D.Sc., University of Manitoba, Winnipeg
New application No. 999
"Effect of smoking on the neonatal EEG and subsequent electrical and functional maturation of the brain".

History

This proposal was case No. 268 (in the name of Victor Chernick, M.D.), and the Executive Committee voted to encourage formal application.

Request

Application No. 999 requests $22,655 plus one additional year.

Documents Submitted

Attached is application dated July, 1974 with CV's of Drs. Havlicek, Haworth, Chernick, and Childeava.

Also submitted was a manuscript "EEG Frequency Spectrum Characteristics Of Sleep States In Full-Term And Preterm Infants" by Drs. Havlicek, Childiaeva, and Chernick; a copy will be sent to you promptly if you wish.

Comment

We shall seek a qualified outside reviewer to evaluate this proposal.

FWN:lb
Encs.

F.W.N.

FWN

1003545565

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

#999

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
JUL 29 1974

Application for Research Grant
(Use extra pages as needed)

Date: July, 1974

1. Principal Investigator (give title and degrees):

Victor Havlicek, M.D., D.Sc.
James C. Haworth, M.B., Ch.B., M.D.
Victor Chernick, M.D.

2. Institution & address:

University of Manitoba
Winnipeg, Manitoba

3. Department(s) where research will be done or collaboration provided:

Department of Pediatrics, Health Sciences Centre
685 Bannatyne Avenue, Winnipeg, Manitoba, Canada

4. Short title of study:

Effect of smoking on the neonatal EEG and subsequent electrical and functional maturation of the brain.

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

1) To determine whether smoking during pregnancy affects brain function at birth as judged by computer analyzed electroencephalography and to correlate the EEG obtained at birth with subsequent intellectual development.

2) To determine whether the prognosis, in terms of physical growth, intellectual development and brain electrical activity of surviving children of women who smoked during pregnancy differs from that of children of non-smoking mothers. Also, to determine whether prognosis can be related to the number of cigarettes smoked or to carboxyhemoglobin content of the blood at birth.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

8. Brief statement of working hypothesis:

The association between cigarette smoking during pregnancy and fetal growth retardation has been firmly established although there may be an element of doubt whether this association is causal. The hypothesis that cigarette smoking interferes with brain growth in-utero and therefore subsequent electrical and intellectual development of the child will be tested in a prospective study.

9. Details of experimental design and procedures (append extra pages as necessary)

The Sample of Pregnant Women

The sample will consist of women attending the Women's Centre of the Health Sciences Centre, where there are nearly 4,000 deliveries a year. In order to interview women at several stages of pregnancy, it would be ideal to include the women who attend the prenatal clinic at the hospital. However these patients tend to be those in the lower socioeconomic catagories and so as to obtain a proper socioeconomic "mix" it will be necessary to include also women who receive their prenatal care at the offices of practising obstetricians. For practical reasons these women can be interviewed only once - while they are in hospital after the delivery of their babies. It is planned that the sample shall consist of 100 clinic patients and 100 postpartum patients.

The following information will be obtained:

1) Smoking History:

Whether a smoker or a non-smoker, the amount of smoking according to the number of cigarettes smoked per day, change in smoking habits during pregnancy

2) Personal and Obstetric Data:

Age, height, pregravid weight, weight gain during pregnancy, ethnic origin, socioeconomic status (rated according to occupation of the woman or the husband) parity, past and present obstetric history.

10035445 67

The Sample of Infants

The following information will be collected about the infants born to the pregnant women studied: birthweight, gestation, crown heel length, head circumference, apgar score (1 minute and 5 minute), presence of major malformations (as defined by the Medical Records Department, Women's Centre), neonatal disorders. Still births and neonatal deaths will also be recorded.

Cord blood carboxyhemoglobin will be measured in as many babies as possible (those born during normal working hours). This estimation will be performed on the

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I.L. CO-Oximeter at the Children's Centre.

The Follow-Up Study

A group of these infants will be selected for the follow-up study. At the moment it is planned to study 100 infants of smoking mothers and an equal number of infants of non-smokers (this is the maximum number that Dr. K. McRae, the Director of the Child Development Clinic, feels that his Department can accommodate). Infants will be selected for follow-up on the following basis:

1) The mother smoked more than 10 cigarettes regularly since the 4th month of the pregnancy.
2) Follow-up is practical (family lives in or near Winnipeg).
3) The mother gives informed consent.

For each index infant selected for follow-up, a control infant will also be selected. The control infant will be the baby born to the next non-smoker who matches the index infant as closely as possible for sex, birthweight and gestational age, and age, parity and socioeconomic class of the mother (all factors which may affect developmental progress). If the mother does not give consent, or if follow-up is impractical, the next born suitable infant will be substituted.

The follow-up procedure for index and control infants will be as follows:

1) Computer analyzed electroencephalogram (EEG) during different stages of sleep in the newborn period. Data on normal infants have already been collected (see appended manuscript). EEG's will be repeated at 6, 12, and 24 months of age.

2) Attendance at the Child Development Clinic at 6, 12 and 24 months of age. The following information will be documented at each visit: a) physical measurements - weight, height, head circumference, b) neurological abnormalities, and c) developmental assessment (Yale Developmental Schedule based on Gesell early developmental testing technique).

Data Handling and Analysis

All data will be coded, punched on to computer cards and analyzed according to standard statistical techniques.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space and facilities to undertake this study are available in the Health Sciences Centre. A fully-equipped experimental EEG laboratory is located adjacent to the newborn nurseries. A special Child Development Clinic has been in operation in the Centre since 1959 and they have agreed to undertake the follow-up studies.

11. Additional facilities required:

Nil

10035445 69

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Dr. V. Havlicek	10%	Nil
Dr. J.C. Haworth	10%	Nil
Dr. V. Chernick	5%	Nil
Dr. Rima Childeava (Professional Assistant) (Dr. Childeava is essential for this project)	100%	$12,000
Technical		
Clerk-Stenographer - This person is essential for the handling of the records. Specifically she will type questionnaire forms, keep records, code the data, arrange for follow-up visits and type correspondence.	100%	$ 5,200
Sub-Total for A		$17,200
B. Consumable supplies (by major categories)		
a) paper, computer punch cards, stenographic supplies		$ 300
b) EEG paper, tape for tape recorder, ink		$ 700
Sub-Total for B		$ 1,000
C. Other expenses (itemize)		
Computer time for analysis of EEG's and statistical work.		$ 1,500
Sub-Total for C		$ 1,500
Running Total of A + B + C		$19,700
D. Permanent equipment (itemize)		
Nil (all necessary equipment available)		
Sub-Total for D		-
E. Indirect costs (15% of A+B+C)		$ 2,955
Total request		$22,655

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$18,500	$1,000	$1,500	--	$3,000	$24,000
Year 3						

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Development of the EEG in the Neonatal Period	Department of National Health and Welfare, Canada		July 1, 1974 to June 30, 1975 (terminal award to complete work on neonatal hypoglycemia and the EEG)

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
NIL			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Drs. V. Havlicek and J. Haworth

Signature V. Havlicek / [illegible] / [illegible] Date

J. Haworth Telephone 204 775-8311 366
Area Code Number Extension

V. Havlicek 204 786-3767

Checks payable to

Mailing address for checks

Responsible officer of institution

Typed Name DR. E. SIRLUCK

Title PRESIDENT UNIVERSITY OF MANITOBA

Signature [illegible] Date JUL 23 1974

Telephone 204 474-9345
Area Code Number Extension

1003544571

SELECTED REFERENCES

1. Astrup, P., Trolle, D. Olsen, H.M., Kjeldsen, K.: Effect of moderate carbon-monoxide exposure on fetal development. Lancet 2:1220, 1972.

2. Becker, R.F. and King, J.E.: Studies on nicotine absorption during pregnancy. II The effects of acute heavy doses on mother and neonates. Amer. J. Obstet. Gynec. 95: 515, 1966.

3. Becker, R.F., Little, C.R.D. and King, J.E.: Experimental studies on nicotine absorption in rats during pregnancy. III Effect of subcutaneous injection of small chronic doses upon mother, fetus and neonate. Amer. J. Obstet. Gynec. 100:957, 1968.

4. Butler, N.R., Goldstein, H. and Ross, E.M.: Cigarette smoking in pregnancy: its influence on birth weight and perinatal mortality: Brit. Med. J. 2: 127, 1972.

5. Butler, N.R., Goldstein, H.: Smoking in pregnancy and subsequent child development. Brit. Med. J. 4: 573, 1973.

6. Cole,P.V. Hawkins, L.H. and Roberts, D.: Smoking during pregnancy and its effects on the fetus. J. Obstet. Gynaec. Brit. Comm.: 79:782, 1972.

7. Comstock, G.W., Shah, F.K., Meyer, M.B., Abbey,H.: Low birth weight and neonatal mortality rate related to maternal smoking and socioeconomic status: Amer. J. Obstet. Gynec. 111: 53, 1971.

8. Cope, I., Lancaster, P. and Stevens, L.: Smoking in pregnancy. Med. J. Aust. 673, 1973.

9. Essenberg, J.M., Schwind, J.V. and Patras, A.R.: Effects of nicotine and cigarette smoke on pregnant female albino rats and their offspring. J. Lab Clin. Med.: 25:708, 1940.

10. Frazier, T.M., Davis, G.H., Goldstein, H. and Goldberg, I.D.: Cigarette smoking and prematurity: a prospective study. Amer. J. Obstet. Gynec.: 81: 988, 1961.

11. Haddon, W. Jr., Nesbitt, R.E.L. and Garcia, R.: Smoking and pregnancy: Carbon monoxide in blood during gestation and at term. Obstet. Gynaec. 18: 262, 1961.

12. Hardy, J.B., and Mellits, E.D.: Does maternal smoking during pregnancy have a long-term effect on the child? Lancet 2:1332, 1972.

13. Kullander, S., Kallen, B.: A prospective study of smoking in pregnancy. Acta. Obstet. Gynec. Scand. 50:83, 1971.

14. Peterson, W.F., Morese, K.M. and Kaltreider, D.F.: Smoking and prematurity. A Preliminary report based on study of 7740 caucasians. Obstet. Gynec. 26:775, 1965.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

16. Russell, C.S., Taylor, R. and Maddison, R.M.: Some effects of smoking in pregnancy. J. Obstet. Gynaec. Brit. Comm.: 73:742, 1966.

17. Russell, C.S., Taylor, R., Law, C.E.: Smoking in pregnancy, maternal blood pressure, pregnancy outcome, baby weight and growth, and other related factors. A prospective study. Brit. J. Prev. Soc. Med. 22:119, 1968.

18. Schoeneck, F.J.: Cigarette smoking in pregnancy. New York State J. Med. 41: 1945, 1941.

19. Simpson, W.J.: A preliminary report on cigarette smoking and the incidence of prematurity. Amer. J. Obstet. Gynec. 73:807, 1957.

20. Sontag, L.W. and Wallace, R.F.: The effect of cigarette smoking during pregnancy upon the fetal heart rate. Amer. J. Obstet. Gynec. 29:77, 1935.

21. Underwood, P.B., Kesler, K.F., O'Lane, J.M. and Callagan, D.A.: Parental smoking empirically related to pregnancy outcome. Obstet. Gynec. 81:988, 1961.

22. Yerushalmy, J.: The relationship of parents' cigarette smoking to outcome of pregnancy--implication as to the problem of inferring causation from observed associations. Amer. J. Epidem. 93:443, 1971.

23. Younoszai, M.K., Kacic, A. and Haworth, J.C.: Cigarette smoking during pregnancy: the effect upon the menatocrit and acid base balance of the newborn infant. Canadian Med. Assoc. J. 99: 197, 1968.

24. Younoszai, M.K., Peloso, J. and Haworth, J.C.: Fetal growth retardation in rats exposed to cigarette smoke during pregnancy. Amer. J. Obstet. Gynec. 104:1207, 1969.

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#902 HEIMSTRA

1003544574

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

May 13, 1974

Grant application No. 982

MISCELLANEOUS

To: The committee comprising Drs. Gardner, Lynch and Sommers

Subject: Norman W. Heimstra, Ph.D., University of South Dakota
New application No. 982
"Effects of Smoking Deprivation on Risk-Taking Behavior"

History

In the past Dr. Heimstra has been awarded several small CTR grants, which were considered to have been productive. Currently he holds CTR grant No. 898 "Effects of Smoking Deprivation on Group Problem Solving"; a progress report on this, just received, is enclosed.

The enclosed new proposal was preceded by telephone conversations between Drs. Heimstra and Hockett.

Request

Application No. 982 requests $10,727 for one year only.

Documents Submitted

Attached is application dated April 30, 1974, with appendices.

Comment

We shall doubtless seek an opinion from an ad hoc consultant.

FWN:gh
Enclosures

F.W.N.

1003544575

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#982

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
MAY 3 - 1974

Application for Research Grant
(Use extra pages as needed)

Date: April 30, 1974

1. Principal Investigator (give title and degrees):
Norman W. Heimstra, Ph.D. Professor and Director, Human Factors Laboratory

2. Institution & address:
University of South Dakota, Vermillion, S.D. 57069

3. Department(s) where research will be done or collaboration provided:
Department of Psychology

4. Short title of study:
Effects of Smoking Deprivation on Risk-Taking Behavior

5. Proposed starting date: Sept. 1, 1974

6. Estimated time to complete: One year

7. Brief description of specific research aims:
To determine whether enforced deprivation from smoking will modify risk-taking behavior.

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

8. Brief statement of working hypothesis:

Smoking deprivation has been shown to modify various affective states of individuals. It is likely that certain aspects of behavior may be associated with these changes in affective state and that smoking deprivation may thus influence these kinds of behavior. It would seem that risk-taking behavior would be particularly susceptible to modification by the stress and changes in affective states brought about by smoking deprivation. The proposed investigation is designed to determine whether this is the case.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached proposal

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space in the Human Factors Laboratory will be available for this study. The test equipment needed to conduct the investigation has been used in a series of previous investigations and is functional. No additional equipment will be needed. Computer facilities in the laboratory are available for data analysis.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached vita

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

100354578

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

Pertinent Publications*

(with C. R. Scoughton) The effects of smoking on peripheral movement detection. Final Report, April 1974, U.S. Army Research and Development Command, Washington, D.C. 20314

The effects of smoking on mood change. In W. L. Dunn (Ed) Smoking Behavior: Motives and incentives. Washington, D.C.: V. H. Winston & Sons, 1973.

(with R. L. Lucas and D. K. Spiegel) Part-task simulation training of driver's passing judgments. Human Factors, 1973, 15, 269-274.

(with N. R. Bancroft and A. R. DeKock) Effects of smoking upon sustained performance in a simulated driving task. In H. B. Murphy (Ed) The effects of smoking on the central nervous system. Ann. N. Y. Acad. Sci., 1967.

(with R. A. Krippner) Effects of smoking on peripheral visual acuity. Technical Report, Human Factors Laboratory, University of South Dakota, 1969.

*Additional relevant studies conducted in my laboratory by members of my staff are listed in the Reference section of the proposal.

1003545529

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

R: REDACTED MATERIAL

14. First year budget:

	% time	Amount
A. Salaries (give names or state "to be recruited")		
Professional (give % time of investigator(s) even if no salary requested)		
Norman W. Heimstra, Ph.D. Principal Investigator		REDACTED
Research assistant (M.A. level graduate student - to be recruited)		REDACTED
Technical		
Hourly assistance 300 hrs. at $2.50/hr.		R
Fringe benefits for above		R
		R
Sub-Total for A		—
B. Consumable supplies (by major categories)		
Maintenance on equipment		$300
Consumable items (paper, questionnaires, etc.)		200
Sub-Total for B		500
C. Other expenses (itemize)		
Payment for subjects (30 at $25 each)		750
Sub-Total for C		750
Running Total of A + B + C		$ 9328
D. Permanent equipment (itemize):		
None		
Sub-Total for D		0
E. Indirect costs (15% of A+B+C)		$ 1399
Total request		$10727

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	None					
Year 3						

100354580

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

5.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Effects of smoking on peripheral movement detection (Year 2)	U.S. Army Medical R&D DADA17-73-7-3037	17,121	11/01/74 - 10/30/75
Deprivation on Group Problem Solving Processes (Year 1)	Council for Tobacco Research #898	12,592	10/01/74 - 9/30/75
Accident Prevention Research (Final year - project to be concluded)	USPHS OH 00002-08	213,500	7/01/69 - 5/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Task Factors Influencing Motivation of Workers	USPHS	28,000	1/01/75 - 12/31/77
Sex differences in response to information overload	Dept. of Army	21,000	1/01/75 - 12/31/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Norman W. Heimstra, Ph.D.

Signature Norman Heimstra Date 4/22/74

Telephone 605-677-5295 (Area Code / Number / Extension)

Checks payable to

University of South Dakota

Mailing address for checks

Business Manager, Univ. of S.D.

Vermillion, S.D. 57069

Responsible officer of institution

Typed Name Richard L. Bowen, Ph.D.

Title President

Signature [signature] Date 4/30/74

Telephone 605-677-5641 (Area Code / Number / Extension)

10035581

Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

INTRODUCTION

Studies concerned with smoking and behavior have typically focused on variables which are considered to be important in determining whether or not a person begins to smoke and other factors that are assumed to be of importance in the maintenance of this type of behavior. Other investigations have dealt with the effects of smoking on certain specific aspects of behavior such as motor skill, learning ability, and so forth. Considerable interest has also been shown in developing techniques aimed at modifying or eliminating smoking behavior. Another area of interest, however, where relatively little research has been conducted deals with the effects on behavior of enforced or voluntary cessation of smoking. The fact that legislative action has resulted in numerous situations where enforced smoking deprivation occurs and that large numbers of individuals attempt to stop smoking on a voluntary basis would indicate that this is an important area of research.

Research in our laboratory has been concerned with this problem for several years. For example, in an early study (Heimstra, Bancroft and DeKock, 1967) which involved subjects operating a driving simulator for a number of hours, smokers who were not allowed to smoke during the time they operated the device performed poorly in contrast to smokers and subjects who were non-smokers. In a series of other investigations, reported in Smoking Behavior: Motives and Incentives (Heimstra, 1973) smoking has been shown to reduce fluctuations in mood states during the performance of stressful tasks while smoking deprivation resulted in increased mood fluctuation. Currently, a study is underway in our laboratory which deals with the effects of smoking and smoking deprivation on group problem solving and social interactions.

While there are some data available, then, from this laboratory and others concerning the effects of smoking deprivation on behavior, there are many types of behavior that have not been studied which may be affected under conditions where smokers are, for various reasons, deprived of smoking. One type of behavior, which we feel may be particularly susceptible to deprivation effects, is that which has been labeled "risk-taking" behavior. Because risk-taking behavior may be determined to some extent by prevailing affective states and, since research has shown that these states are modified under conditions of smoking deprivation, it seems quite likely that risk-taking behavior may also be affected by smoking deprivation.

Risk-taking and decision making behavior has been the subject of a large amount of research but the emphasis has been on the development of subjective probability models and abstract decision theory (Coombs, Dawes, Teversky, 1970; Edwards and Tversky, 1967; Edwards, 1968; Kogan and Wallach, 1964). Actually, very little research has been conducted where this type of behavior was studied in "real-world" settings or even in simulated real-world settings. In our laboratory, as part of the ongoing

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research program concerned with driver behavior, we have developed a methodology for studying risk-taking behavior in a laboratory setting where certain aspects of the driving task are simulated. While these tasks are described in detail in the METHODS section of this proposal, a brief overview of the type of research that has been conducted with these tasks will be given at this point.

Both of the tasks can be considered as laboratory analogs of the automobile passing task. One of the tasks was initially developed (DeKock, 1968) to study the relationship between decision making under conditions of risk and selected psychological tests while the other task was designed initially to train drivers on a critical passing skill (Lucas, Heimstra, and Spiegel, 1973). These tasks have been used in a variety of studies in the applicant's laboratory. For example, under a contract from Abbott Laboratories (Ellingstad, Struckman and Sebring, 1972) the effects of a new drug (Abboutt 35616 - Tranxene) on performance on these tasks was determined. In another study, funded by the Department of Transportation (Ellingstad, McFarling and Struckman, 1973), the effects of marijuana and alcohol on performance on these tasks was investigated. Recently, a study has been completed which compared performance on the laboratory passing task while subjects were intoxicated with performance in an actual vehicle with the same subjects again intoxicated (Stone and Ellingstad, 1974). Several other unpublished exploratory studies have also been conducted with these tasks. In general, then, these tasks have been shown to be sensitive to several types of independent variables and to different levels of these variables.

The objective of the proposed investigation is to determine whether "risk-taking" behavior, as measured by these tasks, will be modified when smokers perform on these tasks after a period of smoking deprivation.

METHODS

Subjects:

Subjects will be 30 male volunteers from the University of South Dakota student population. All will be solicited from the "pool" of Ss obtained at the beginning of each semester on the basis of questionnaires administered to students in several large classes dealing with their smoking habits. All Ss will be "smokers" i.e., individuals who smoke at least 20 cigarettes a day and have done so for at least six months. All potential Ss are screened for visual defects prior to being accepted for the study. Ss are paid to participate.

The 30 Ss will be randomly assigned to two groups of 15 Ss each. One group will be tested under a "smoking deprived" condition and the other group under a "smoking" condition.

Task and Apparatus:

Film Passing Task: The Film Passing Task apparatus consists of two sequences of 16mm color film presented on a rear projection screen to a subject seated in a mock-up of an automobile cockpit in an adjoining room. Figure 1 depicts the physical

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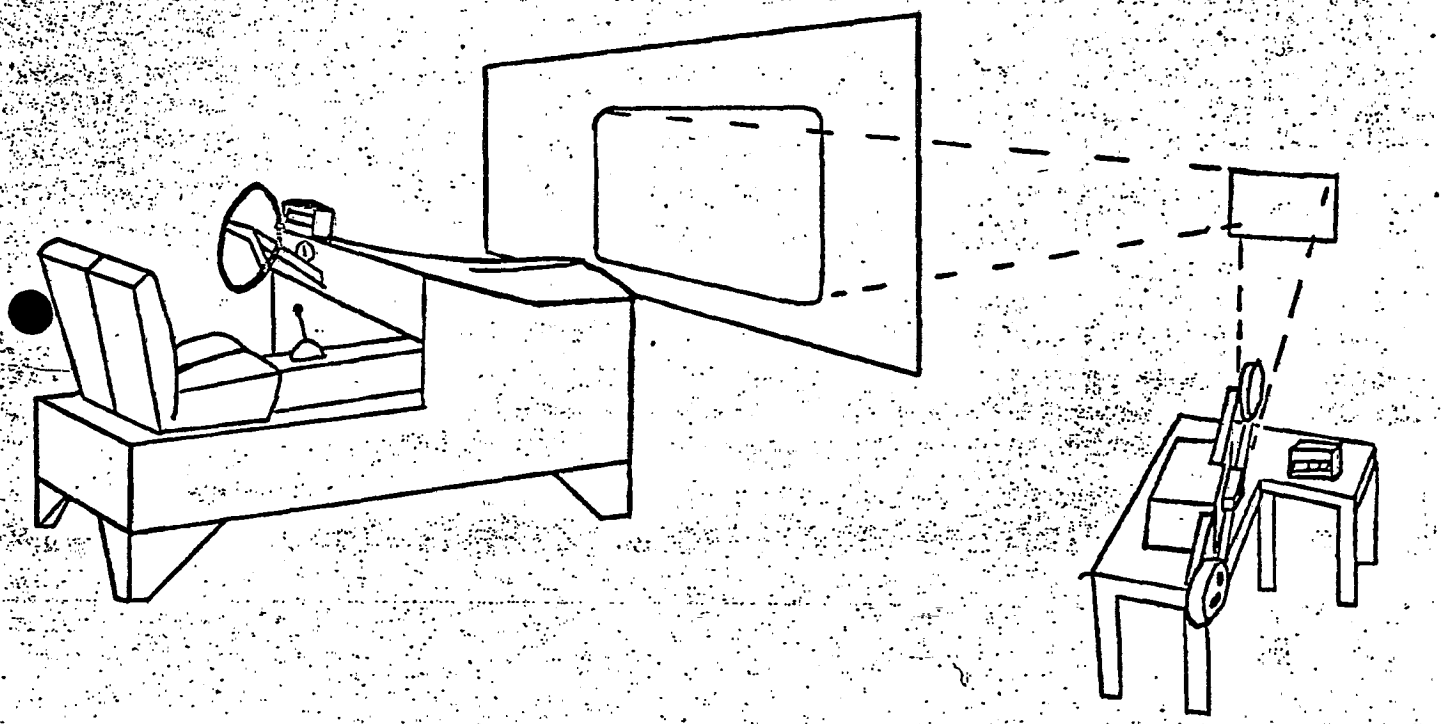

Figure 1

Film Passing Task Projection System

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setup of this task apparatus.

The first of the film sequences, the passing film, was taken from the driver's position in a vehicle following a "lead car" maintaining a constant speed of 60 miles per hour on a four-lane divided highway. The car following the lead car will henceforth be referred to as the "passing car." The passing film was divided into 47 scenes in which the passing car would begin behind the lead car, overtake the lead car, and return to the right lane of travel. The passes were accomplished in a manner such that each pass was completed in the shortest time possible. An effort was made to maintain a consistent passing time between scenes. The second of the sequences, the oncoming film, was filmed on two-lane highways with the passing car remaining behind the lead car throughout the duration of the film. Naturally occuring traffic served as oncoming vehicles in the respective scenes. Each scene begins with the passing vehicle following the lead car on the highway. Shortly after the scene begins, a vehicle in the oncoming lane appears at a distance that allows the subject sufficient time to notice the oncoming vehicle. The scene proceeds with the oncoming vehicle gradually approaching the lead car, coming abreast of the lead car, and passing on. Thirty-five of these scenes comprise the oncoming sequence of the film passing task.

The sound track of the oncoming film is modified in order to provide a consistent automatic timing system for computing the subject's errors. For each of the 35 scenes in the oncoming film, a small hole has been drilled in the sound track opposite the frame which represents the last possible safe moment to pass for that particular scene. The placement of this hole was determined by computing the mean time required to pass by the passing vehicle in the production of the passing film. Time required to pass for each scene is defined as the time elapsed from that point when the left wheels of the passing vehicle crossed into what would be the opposing lane of traffic on a two-lane highway to the time when the left wheels of the passing vehicle entered the proper traffic lane after accelerating around the lead car, thus completing the pass. The mean time required to pass was then multiplied by the film speed in frames per second, to determine the number of frames in advance of the frame in which the oncoming car came abreast of the lead car, thereby locating the placement of the hole and defining the last safe moment to pass for that particular scene.

The Film Passing Task utilizes the first sequence, or the passing film sequence, to familiarize the subject with the capabilities of the passing vehicle. The subject views the passing film to obtain an idea of how quickly the lead car could be passed by the passing vehicle. The oncoming sequence of the Film Passing Task is used to establish the measures of risk-taking used in the investigation. After viewing the passing film sequence of the task, the subject is instructed to indicate the last possible moment he could begin and successfully complete a pass of the lead car without colliding with the oncoming vehicle in the oncoming film scenes. The subject's

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decision is based on the information he had received from the passing film sequence. The subject indicates his decision as to the last safe moment to pass by pressing a hand-held response switch. Provision is made for feedback to the subject via a digital readout display. The display provides the subject with information as to the magnitude and direction of his errors. Direction of the error is defined as either early or late. An early response indicates the subject had decided to pass at a point in time such that, had he actually initiated the pass, he would have been able to successfully complete the pass with time to spare. A late response, on the other hand, indicates that the subject had responded in such a manner that he could not have successfully completed the pass; rather, his decision to pass would have resulted in forced evasive action or possible collision. The magnitude of the error is presented in seconds early or late (indicated by plus and minus signs, respectively) to the nearest hundredth of a second. This information is also presented to the experimenter on a separate digital display.

Performance Measures - Film Passing Task: As indicated, a Ss score for each scene in the oncoming film consisted of a direction (early or late) and a magnitude in seconds. These scores are broken down to derive the following performance measures.

Mean constant error of estimate: This measure represents the simple mean of the algebraic sum of the S's errors of estimate. The measure reflects both direction and magnitude of the S's errors. It is indicative of the subject's average magnitude of error, without regard to direction.

Root mean square error or estimate: This is a measure of the subject's variability about his own mean error of estimate. It reflects a subject's consistency of error, with respect both to direction and magnitude.

Ratio of late responses to total trials: This measure is indicative of the subject's tendency for late error estimates.

Passing/Decision Task: The Passing/Decision Task apparatus consists of two sets of two clocks each, a clear incandescent light bulb, one yellow indicator lamp, one red indicator lamp, and the subject's response switches Figure 2 is a schematic diagram of the display. Each set of clocks contains one three-hand Standard Electric Timer and one digital readout elapsed-time clock. The Standard Timers are incorporated to elicit a sense of motion or the active passage of time. The digital clocks are used by the subject to obtain an indication of the time elapsed in the task, and his accumulated progress. The subject's task is to keep to a minimum the elapsed time difference between the two digital clocks. One set of clocks represents "real time" or the ordinary passage of time. These clocks run throughout the duration of the task.

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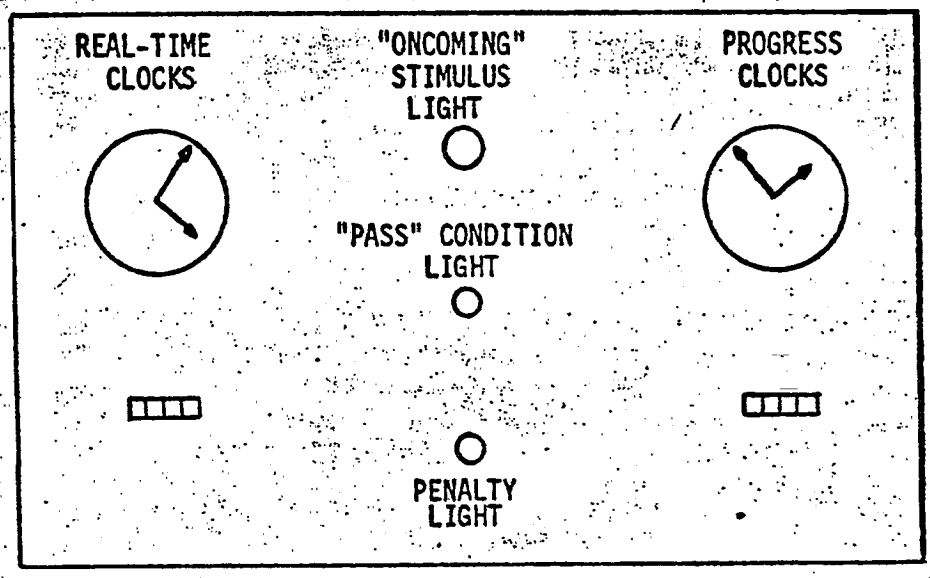

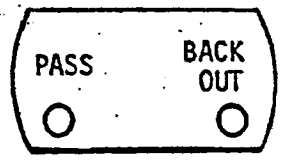

SUBJECT'S RESPONSE SWITCHES

Figure 2

Passing/Decision Task Apparatus Schematic

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The other set of clocks represent the progress of the subject's vehicle on a hypothetical journey. When the subject's "progress clocks" are running, the subject's vehicle is proceeding on the journey unimpeded. When the clocks stop, the subject's vehicular progress is said to have been impeded by a slow-moving vehicle in his lane of traffic. Once the subject's progress clocks stops the clear light comes on at some initial intensity, continues to get brighter until it reaches a fixed intensity, and extinguishes. This clear light represents an oncoming vehicle. The greater the intensity of the clear light, the closer the oncoming vehicle is considered to have been to the subject's vehicle. The initial intensity of the clear light varies for each trial, thus representing different distances from the subject's vehicle, or different amounts of time in which to complete a pass (8, 9, 10, 11 and 12 seconds). Once the clear light comes on, the subject has two options. The first option entails waiting until the clear light reaches its terminal intensity and extinguishes, at which time the progress clocks restart. The subject's second option is to initiate a "pass" by pressing his pass response switch. This action restarts the subject's progress clocks, as well as illuminating the yellow light, signifying that he is in the pass condition. Once the subject elects to pass, a fixed time of 10 seconds is required to complete the pass. Considering the different times of 8, 9, 10, 11 and 12 seconds specifying the pass conditions, and the fixed time of 10 seconds required to complete a pass, it becomes evident to the subject that some trials allow him to safely initiate and complete a pass, while other trials do not allow enough time to complete a safe pass. The actual time required to complete a pass or the times in the different passing trials are never explicitly mentioned to the subjects, however. Thus, if the fixed time required to pass elapsed before the clear light was extinguished, the yellow light will go out and his progress clocks will continue to run, indicating to the subject that his pass attempt has been successful. If the clear light reached its final fixed intensity and extinguishes before the time required to pass has elapsed, the progress clocks will stop and the red light will come on, indicating to the subject that his attempted pass has been unsuccessful, or that he has incurred a collision. Upon the occurence of a collision, the subject's progress clocks remain stopped for a period of 30 seconds as a collision penalty.

Once the subject has opted to pass, however, he can reverse his decision if, in his judgment, the pass cannot be successfully completed. If the subject does decide to reverse his decision, he responds by pushing his "back out" response button. This action stops the progress clocks, and turns off the yellow passing indicator light. An additional cost factor is built into the back-out decision, however, in that once the subject decides to reverse his decision or "back out", he will remain in the back-out condition for a period of time equal to the time that had elapsed in the pass condition. Thus it is

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possible for a collision to occur even though the subject had elected to reverse his initial decision to pass and subsequently backed out. Each passing condition is presented in six trials, in a random order, for a total of 30 trials per task session. A flowchart of subject and apparatus logic decisions appears in Figure 3.

Performance Measures-Passing/Decision Task: In this task Ss make at least one decision and possibly two decisions per trial. The cumulative results of these decisions, in addition to the time factor involved for the initial decision of each trial, are interpreted in terms of the following seven measures:

Total number of safe passes: This measure reports the number of successful decisions to attempt a complete pass on the part of the subject.

Total number of safe backouts: This is a measure of the subject's number of successful decision reversals, once he had elected to pass during a trial and then, due to perceived or actual misjudgment or as a result of initial hesitancy, decided to abort the attempted pass.

Total number of pass "collisions": This is a cumulative measure of unsuccessful attempts to complete a pass.

Total number of backout collisions: This is representative of unsuccessful decision reversals, once an attempted pass had been initiated.

Total number of no responses: This measure reports the number of trials in which the subject did not initiate a passing attempt.

Mean decision latency time: This is the average time elapsed before the subject decided to initiate an attempted pass, whether or not the attempt was successful.

Time difference: This is the difference between the elapsed times on the real-time clock and the subject's progress clock. This measure was taken at the end of the task session.

As pointed out previously in the introduction to this proposal, both of the tasks described above have been used in a number of investigations in the applicant's laboratory. They have been found to discriminate between control groups and groups which have been exposed to several types of drugs. The Passing/Decision Task has also been used in the USPHS Injury Control Laboratory in a series of alcohol/driving studies. In general, we feel that both of these tasks "get at" a type of behavior that has been proved to be elusive in the past and which may be a critical type of behavior not only in the driving task but also in many other kinds of situations.

Data Analysis

The principal statistical technique to be utilized in the analysis of data from the proposed investigation will be a multiple discriminant analysis. The multiple discriminant

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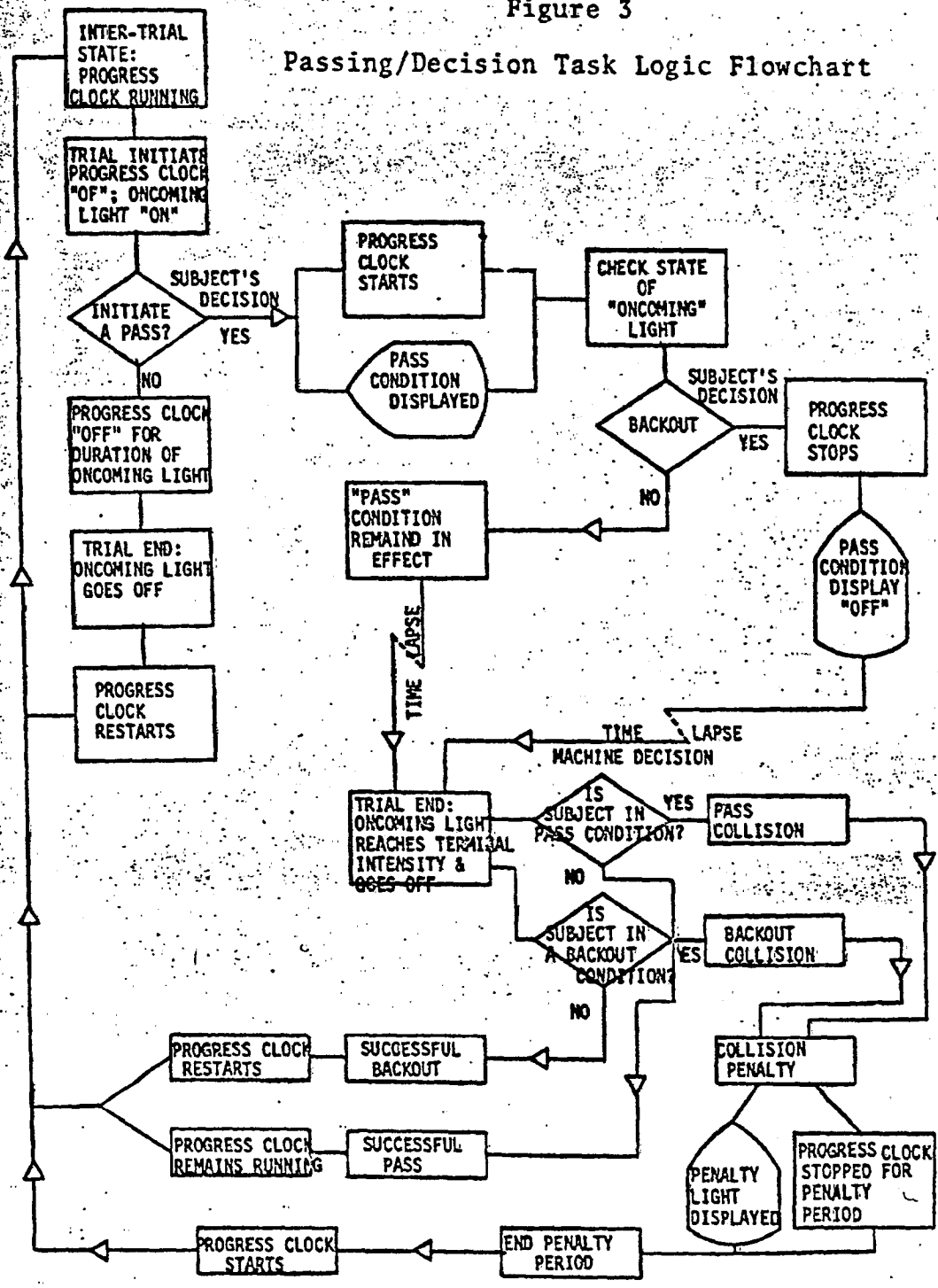
Figure 3

Passing/Decision Task Logic Flowchart



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analysis represents the relatively straightforward extension of the simple one-way univariate analysis of variance to the situation in which multiple dependent variables must be used to characterize the performance of subjects. In the present instance, a total of 11 dependent variables, four from the Film Passing Task and seven from the Passing/Decision Task, will be used to describe the performance of subjects. The multiple discriminant analysis simultaneously evaluates the battery of dependent variables establishing, through the application of the principal components method, a series of sets of discriminant function weights which may be applied to the individual scores on each variable in order to yield a single score which is a linear combination of the 11 dependent variables. A total of n or k-1 (whichever is less) orthogonal linear combinations may be obtained in this analysis, where n equals the number of variables and k equals the number of groups to be deiscriminated. The statistical criterion in the determination of discriminant function weights is to maximize between groups with respect to within-groups variance. Thus the first discriminant dimension(first linear combination of dependent variables) will ordinarily account for the greatest amount of variability and will represent the greatest separation between the groups. Each successive dimension is orthogonal to the remaining dimensions and will ordinarily account for a smaller proportion of total variance.

The interpretation of the results of the multiple discriminate analysis involves an inspection of the set of discriminate function loadings obtained for each separate significant dimension on which between-groups discrimination is possible. The loadings represent the correlation of each variable with the linear combination obtained as a result of the application of the discriminant function weights to the raw data, and as such reflect the importance of each separate variable to the linear composite on the basis of which discrimination was possible.

Procedures

Subjects will report to the laboratory at 9 A.M. (six hours prior to the actual testing). During most of this six hour period, Ss remain in a comfortable waiting room equipped with reclining chairs, TV, magazines, etc. Subjects assigned to the "smoking-deprived" group are not allowed to smoke during this period while Ss assigned to the "smoking" group are allowed to smoke.

Due to the sensitivity and complexity of the two tasks that are utilized, all Ss are given practice sessions on the tasks during the six hour waiting period. The use of practice sessions will serve to familiarize the Ss with the nature of the tasks and will also break up the monotony of the waiting. Practice sessions in the Passing/Decision Task will consist of the reading of detailed and comprehensive instructions to insure the Ss understanding of the task. Upon completion of the instructions the task is presented in the same manner that it will be presented in the actual test session. Practice sessions in the Film Passing

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Task will also consist of instructions and the presentation of the task. Task order will be counterbalanced both between and within subjects in both the practice and actual test sessions. Instructions and practice will be given between 9A.M. and noon. After the practice sessions, Ss will return to the waiting room and will be furnished with a light lunch. Actual testing will begin at 3 P.M. Testing on both tasks requires slightly over one hour. During the testing, Ss in the deprived group are not allowed to smoke while Ss in the smoking condition are permitted to smoke.

Significance of the Research

Previous research dealing with the effects of smoking deprivation has shown that deprivation may have an effect on certain aspects of performance as well as a modification of the affective state of an individual. Because smoking deprivation, either enforced or voluntary, is a common condition and becoming even more common because of legislative action, it would appear that more data concerning the behavioral effects of deprivation is urgently needed. Anecdotal data, as well as some evidence from studies dealing with mood states and smoking deprivation, suggest that deprivation may induce aggression and other affective states that may have an effect on decision making and risk taking behavior. Data supporting this assumption, however, are lacking. The proposed investigation would determine whether deprivation does, in fact, have an effect on this type of behavior as measured by two test systems which have demonstrated their effectivenss in a variety of studies. It seems to the applicant that it is important to systematically study the effects of smoking deprivation on a variety of types of behavior in order to be able to objectively inform legislative groups and others about possible negative effects of banning smoking in legislative sessions, committee meetings, during operations of various types of equipment, and so forth.

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VITA

Norman W. Heimstra

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5. Research Scientist - HumRRO 1960-1961.
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VI. Academic Experience

Courses Taught--Introductory Psychology
Physiological Psychology (undergraduate)
Seminar in Physiological Psych. (graduate)
Seminar in Psychopharmacology (graduate)
Industrial Psychology (undergraduate)
Personnel Selection & Training (graduate)
Human Factors Psychology (graduate)
Seminar in Accident Prevention Research (graduate)

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Truman M. Mast (1966) Influence of motivational variables on prerest and postrest performance in rotary pursuit tracking

Norris Bancroft (1968) Relationship between smoking and psychophysiological response to stress

Mark Hofmann (1968) A comparison of visual, auditory and electrocutaneous displays in a compensatory tracking task

Arlan DeKock (1968) Relationship between decision making under conditions of risk and selected psychological tests

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David Damkot (1969) A comparison of auditory, visual, and electrocutaneous displays in a vigilance task

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Richard Lucas (1970) Development and evaluation of a part task film simulation technique for training drivers on a critical passing skill

Thomas R. Schori (1970) A comparison of visual, auditory, and cutaneous tracking displays when divided attention is required to a cross-adaptive loading task

Harold D. Warner (1970) Effects of intermittent noise on visual search tasks of varying complexity

Kent Kimball (1971) Combined task performance: A study of the effect of coded signal processing on a compensatory tracking task

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Leslie H. McFarling (1974) An investigation of noise effects, sex differences, and product presentation in simulated inspection

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VIII. Research Support

Approximately one million dollars since 1963.

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

August 7, 1974

Grant application No. 1007

MISCELLANEOUS

To: The committee comprising Drs. Gardner, Liebow, and Wyatt

Subject: Edwin A. Kroeger, Ph.D., University of Manitoba, Winnipeg
New application No. 1007
"Effect of Nicotine on Respiratory Airway Smooth Muscle and Pregnant Myometrium: Biophysical, Biochemical and Electrophysiological Studies".

History

A preliminary inquiry was handled as case No. 262 and the Executive Committee voted to encourage formal proposal.

Request

Application No. 1007 requests $27,508 plus two additional years.

Documents Submitted (attached)

1. Application dated July 30, 1974.

2. Reprints of papers listed on page 3c of the application as numbers 1, 3, and 9.

3. Manuscript of the paper checked on page 3d of the application.

F.W.N.

FWN:lb
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P-1007

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

RECEIVED
AUG 6 1974

Application for Research Grant
(Use extra pages as needed)

Date: July 30, 1974

1. Principal Investigator (give title and degrees):

Edwin A. Kroeger, Ph. D.
Assistant Professor

2. Institution & address:

Department of Physiology
Faculty of Medicine
University of Manitoba
770 Bannatyne Avenue
Winnipeg, Manitoba, R3E 0W3

3. Department(s) where research will be done or collaboration provided:

Department of Physiology

4. Short title of study:

Effect of Nicotine on Respiratory Airway Smooth Muscle and Pregnant Myometrium: Biophysical, Biochemical and Electrophysiological Studies

5. Proposed starting date: January 1, 1975.

6. Estimated time to complete: Three Years

7. Brief description of specific research aims:

Pregnant myometrium (Years 1 & 2)

The proposed research is to be conducted within the context of my ongoing research program on the mechanisms whereby the crucial modification of function of the pregnant uterus is effected at term (i.e. from a relatively quiescent organ to one producing powerful co-ordinated contractions). The specific aim of the proposed research is to investigate mechanisms which might be responsible for the premature delivery of the fetus, a phenomenon which occurs with increased frequency in smoking mothers. In investigating these mechanisms the following possibilities will be considered (see rationale below, section 8): 1. that nicotine affects the production of prostaglandins, the sensitivity of the myometrium to prostaglandins and/or the potentiation by prostaglandins of uterine responses to oxytocin; 2. that nicotine alters the release of acetylcholine in the myometrium; 3. that nicotine affects tissue cyclic AMP content (cyclic AMP has been implicated in mediating inhibition of myometrium); 4. that nicotine affects the electrophysiological properties of the myometrium. Specific experiments utilizing biophysical, biochemical and electrophysiological tools to study the possibilities are detailed below (section 9).

Airway Smooth Muscle (Year 3)

The following studies are proposed within the context of my ongoing research on mechanisms of tone in airway smooth muscle. The specific aims of the proposed research are 1. to document the effects of nicotine on the biophysical and electrophysiological characteristics of canine tracheal smooth muscle (this preparation has the advantage that it is not subject to the uncontrolled and interacting variables present in tracheal ring chains and whole animal preparations on which most previous studies have been conducted); 2. to determine whether all of the effects of nicotine can be attributed to neural mechanisms and; 3. to study the effect of nicotine on the biophysical and electrophysiological effects of tetraethylammonium (TEA) on tracheal smooth muscle. Since TEA converts trachealis muscle from a tonic, multi-unit muscle to a spontaneous phasic single-unit muscle exhibiting action potentials and a myogenic response, the mechanisms underlying these changes and their modification by nicotine could have wide physiological significance. (Details of the rationale and experimental protocol follow in sections 8 and 9 below).

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8. Brief statement of working hypothesis:

Pregnant Myometrium

Recent evidence has linked the activity of the pregnant uterus at term to direct and indirect effects of prostaglandins. Vane and Williams (1973) have shown that 1) the spontaneous activity of isolated term-pregnant rat uterus is inhibited by agents which inhibit prostaglandin synthesis 2) this activity is restored by the addition of PGE_2 and $PGF_{2\alpha}$ to the bathing solution, and 3) the release of $PGF_{2\alpha}$ from the uterus is greatly increased on the day of expected parturition. In addition Karim and Sharma (1971) and Gillespie et al (1972) have adduced evidence for an effect of prostaglandins on oxytocin release from the posterior pituitary in humans and Gillespie (1972) has shown that $PGF_{2\alpha}$ and PGE_2 potentiate the action of oxytocin. Thus, in addressing research to the well documented problem of premature parturition in smoking mothers (Herriot et al, 1962; Underwood et al, 1965; Mulcahy, 1968; Murphy & Mulcahy, 1971) the relationship of the pregnant uterus to prostaglandins and oxytocin would appear to be a key controlling factor of interest. Specifically, the hypothesis to be tested is that nicotine alters the relation of the uterus to prostaglandins and oxytocin so as to produce premature parturition. This might be accomplished by 1) an increased synthesis of prostaglandins 2) an increased sensivity of the myometrium to prostaglandins and/or oxytocin 3) decreasing the effect of prostaglandins on cyclic AMP production, since cyclic AMP has been implicated in inhibition of the myometrium. An effect of nicotine on the release of acetylcholine will also be investigated.

Another factor to be considered in the future pending the outcome of these experiments is the effect

(Continued Appendix 1)

9. Details of experimental design and procedures (append extra pages as necessary)

I Experimental Design

A. Pregnant Myometrium

Since the development of coordinated, strong contractions in term-pregnant myometrium has been related to 1) an increase in the release of prostaglandins E_2 and $F_{2\alpha}$ (Karim, 1968) 2) an increase in the ratio of $PGF_{2\alpha}$: E_2 in labor (Karim, 1968; Liggins, 1973) 3) a potentiation of the effect of oxytocin by $PGF_{2\alpha}$ and E_2 (Gillespie, 1972) as well as 4) extra-uterine effects involving a stimulation of oxytocin release from the posterior pituitary by $PGF_{2\alpha}$ and E_2 (Gillespie et al, 1972) and an effect of these prostaglandins in producing progesterone withdrawal (de Paiva and Csapo, 1973), an evaluation of the mechanism of premature parturition must take into consideration these various influences. The present study focuses on the effect of nicotine on the relationship of prostaglandins and oxytocin to term pregnant uterus. In order to study these effects in a comprehensive way the present proposal involves a multidisciplinary approach utilizing biophysical, biochemical and electrophysiological techniques, all of which are in routine use in my laboratory. The following specific experiments are proposed:

Biophysical

1) Measurement of the effect of nicotine on the basal tone and spontaneous activity of isolated, term pregnant rat myometrium in the presence and absence of atropine and inhibitors (indomethacin, meclofenamate) of prostaglandin synthesis. These experiments are designed to determine the presence of a cholinergic component in the response and to document in detail the mechanical response of our preparation to these agents. This is of particular importance since our recent studies (Marshall & Knoeger, 1973) have shown that the basal tone maintained by the uterus between spontaneous contractions is largely supported by active mechanisms and our as yet unpublished observations show that this basal tone changes qualitatively with the progress of pregnancy. While its relation to the control of uterine function is not yet clear it may be an index of subtle changes effected in the myometrium with advancing pregnancy and should be documented more thoroughly in parallel with observations on the effect of nicotine on the magnitude of contraction.

2) Measurement of the effect of nicotine on the dose-response relation to oxytocin and prostaglandins E_2 and $F_{2\alpha}$ and the potentiation of the effect of oxytocin by the prostaglandins. These experiments should provide a powerful tool in the analysis of the effect of nicotine on both the direct effects of these uterine stimulants as well as their interactive effects.

(Continued on Appendix 2)

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APPENDIX 1

8. (C)f nicotine on progesterone-withdrawal caused by prostaglandins (de Paiva and Csapo, 1973)

Airway Smooth Muscle

The realizations that the calibre of airways is actively regulated by smooth muscle and that this regulation has usually been studied in preparations subject to a variety of uncontrolled and interacting factors (e.g. whole animal preparations, tracheal ring chain Hawkins & Paton, 1958; Widdocombe, 1966) has stimulated my interest in this smooth muscle. We (Stephens et al, 1969; Stephens and Kroeger, 1970; Kroeger and Stephens, 1971) have chosen the canine trachealis muscle preparation as an eminently suitable model for respiratory airway smooth muscle. My recent studies have shown that tetraethylammonium (TEA) produces phasic electrical and mechanical activity in isolated tracheal smooth muscle which is normally quiescent and produces only tonic responses to stimulating agonists. Since both TEA and nicotine have been shown to affect cholinergic neural elements in other preparations, it would be of interest to study the effect of nicotine on this musale directly as well as its influence on TEA-induced responses. Thus the hypothesis to be tested is that the biophysical and electrophysiological effects of nicotine on airway smooth muscle can be attributed to neural mechanisms. The use of TEA as a tool in producing phasic electrical activity should be an invaluable tool in unmasking effects of nicotine on the muscle membrane.

APPENDIX 2

9. (B)iochemical

1. Measurement of the effect of nicotine on the release of specific prostaglandins. These experiments are designed to test the hypothesis that nicotine alters the synthesis of prostaglandins in the uterus and that the various prostaglandins are affected differentially. In order to obtain some further insight as to the relative importance the decidua and the myometrium in these effects, these structures will be studied independently.

2. Measurement of the effect of nicotine on tissue cyclic AMP levels and on the response of cyclic AMP levels to β-adrenoceptor activation (by isoproterenol). Since a large number of studies have suggested an inhibitory influence of tissue cyclic AMP (Kroeger and Marshall, 1974; Marshall and Kroeger, 1973; Anderson 1973, Triner et al, 1972) and tissue levels as cyclic AMP are very sensitive to stimulatory agonists, being decreased very rapidly (Kroeger & Moorhouse, 1974) these studies should provide a very sensitive index of effects of nicotine on the adenyl cyclase-phosphodiesterase system in whole tissue.

Electrophysiological

1. Measurement of the effect of nicotine on the resting membrane potential and on the height of action potential.

2. Measurement of the effect of nicotine on the cable properties (membrane resistance, length constant) (M)yometrium.

(Continued on page 2b)

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APPENDIX 2 CONT'D

The measurement the various electrophysiological parameters mentioned above (which, in practise, can all be measured in the same preparation using a Tomita bath, see Procedures below) should permit an analysis of electrophysiological mechanisms of the biophysical effects observed. This data yields evidence concerning membrane permeability, changes in Na and K gradients and changes in conduction velocity, all of which might be involved in mediating the biophysical response.

B. Airway Smooth Muscle

(The following is a brief overview of experiments planned for the latter part, year 3, of the proposed research)

Isolated canine tracheal smooth muscle is of the multi-unit variety (Kroeger and Stephens, in press; Stephens et al, 1969) in that it 1) exhibits no spontaneous mechanical or electrophysiological activity 2) responds to stimulation with graded depolarization and tonic responses and 3) its responses to electrical field stimulation are mediated via intrinsic neural elements. However, our recent finding that in the presence of tetraethylammonium (TEA) the trachealis takes on the properties of single-unit smooth muscle ("spontaneous" phasic electrical and mechanical activity, myogenic response to stretch) suggests that the function of tracheal muscle can be modified quite simply. Hence any understanding of the effect of nicotine on respiratory airways must take into account its effect on both the multi- and single-unit characteristics of this smooth muscle, this phenomenon having been previously overlooked. Previous studies on the effect of nicotine on airway smooth muscle have been carried out in the tracheal ring chain preparation (Hawkins & Paton, 1958) which permits only a gross evaluation of smooth muscle performance since 1) the muscle is subjected to the elastic stress of the cartilaginous ring and 2) the oxygenation of the muscle is impaired due to the presence of the mucosa on one side. While these studies did show neural components to the responses seen, they were not designed to discern effects of nicotine on airway smooth muscle itself. Since our recent evidence suggests that the effect of TEA is on the muscle membrane it seems possible that another ganglionic agent, nicotine may also act on the muscle directly. Thus, in view of this new data, it would be of interest to study the effect of nicotine on the single-unit as well as the multi-unit characteristics of tracheal smooth muscle.

The following experiments are proposed in order to document the effect of nicotine on the multi- and single-unit (with TEA) properties of the trachealis and to differentiate between its actions on neural elements and the muscle per se

Biophysical

1. Measurement of the effect of nicotine on basal and carbachol-induced tone in the trachealis in the presence and absence of atropine and hexamethonium.

2. Measurement of the effect of nicotine on the stimulus-response (electrical field stimulation) relation in the presence and absence of atropine and hexamethonium.

3. Measurement of the effect of nicotine on the mechanical response to TEA, including the dose-response relationship, in the presence and absence of atropine and hexamethonium.

The first two of the above experiments are designed to detect an effect of nicotine on the resting and active properties of this muscle in its multi-unit mode and to define or unmask any neural component of this effect. The third experiment is designed to document the effect of nicotine in the single-unit muscle and to define any cholinergic component in the response.

(Continued on page 2c)

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APPENDIX 2 CONT'D

Electrophysiological

1. Measurement of the effect of nicotine on the resting membrane potential in the presence and absence of atropine, hexamethonium and TEA.

2. Measurement of the effect of nicotine on the cable properties, membrane resistance and rectification of tracheal muscle in the presence and absence of atropine, hexamethonium and TEA.

3. Measurement of the effect of nicotine on action potentials stimulated in the presence of TEA.

The above experiments are designed to provide a thorough documentation of the effect of nicotine on the electrophysiological properties which could modify the function of single-and multi-unit trachealis smooth muscle.

II Methods of Procedure

A Biophysical Studies

1. Dissection

(a) Myometrium

Term-pregnant Sprague-Dawley rats are used within one day of expected parturition and strips of myometrium obtained according to methods which we have published previously (Kroeger & Marshall, 1973, enclosed). Briefly, the rats are anesthetized with diethyl ether, the uterus removed and the fetuses gently expelled. Longitudinal segments of the anti-mesometrial edge of the uterus are then prepared and placed immediately for equilibration in a Krebs-Ringer solution. The composition of this solution is as follows (in mM): NaCl, 118.5; $NaHCO_3$, 23.8; KCl, 4.7; KH_2PO_4, 2.4; $MgCl_2$, 1.0; $CaCl_2$, 2.5; dextrose 5.5; this is bubbled with a gas mixture containing 95% O_2 and 5% CO_2 so as to obtain a pH of 7.38 at 37°C.

The advantages of this preparation are that 1) it is stable in its biophysical and electrophysiological properties for a number of hours after pre-equilibration, and 2) it contains a good longitudinal muscle layer and a minimum of endometrium.

(b) Trachealis Smooth Muscle

Mongrel dogs, 7-10 Kg in weight are anesthetized with 30 mg/kg sodium pentobarbital I.V. The cervical trachea is then removed and placed immediately in a Krebs-Ringer solution (as above). The dissection is carried out as previously described (Stephens et al, 1969). Briefly, a single ring of the trachea is cut free, the cartilage bisected and the section everted. The mucosa is then easily separated from the muscle layer and removed. The muscle is then tied at its insertions to the cartilage, cut free and placed for pre-equilibration in a Krebs-Ringer solution at 37° C.

The advantages of this preparation are 1) it is very stable in its mechanical and electrophysiological properties for a period of hours after pre-equilibration 2) it contains less than 25% connective and non-muscle tissue 3) muscle fibres are all oriented in the same general direction (parallel-fibred) 4) it can be tetanized and 5) its length-tension diagram shows a clearly defined optimal length at a low resting tension (Stephens et al, 1969).

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APPENDIX 2 CONT'D

2. Recording of Isometric Myograms (from uterus and trachealis)

The lower end of the muscle is tied with 3-0 braided surgical silk to a hook at the bottom of a bath. The upper end is attached to a force transducer (Statham) which is connected to a bridge amplifier and dynograph recorder (Brush, Mark 280). The bath, which is maintained at 37 ± 0.5° C and is constantly bubbled with a 95 - 5% O_2 - CO_2 mixture can be rapidly drained and refilled with fresh solution to remove drugs after responses are complete. The force transducer is mounted on a rack and pinion so as to adjust the length of the muscle as desired. Field stimulation is effected through platinum plate electrodes from a 60Hz source.

3. Measurement of dose-response relationships

All studies are conducted at the optimal length of the muscle. This is determined by stimulating the muscle with a supramaximal field stimulus at various lengths and the length-tension diagram plotted. Force is normalized as grams/cm^2 and the length at which the muscle develops the greatest active tension (resting tension subtracted from total tension) is considered to be the optimal length.

The effect of nicotine on the dose-response relation of myometrium to oxytocin and prostaglandins will be determined as follows: The dose-response relation of the myometrium to oxytocin and prostaglandins will first be determined by adding increasing amounts of these agents to the bath in separate exposure intervals and monitoring the myogram for a standard (1 minute) interval. The bath will be washed after each test and the muscle allowed to re-equilibrate 5 minutes before the next dose is tested. This protocol should prevent desensitization and provide a stable muscle preparation. Since the responses to these agents will not be tonically sustained but will include increases in "spontaneous" phasic activity, the response will be quantified in terms of the area under the myogram (as measured by planimetry). Potentiation of oxytocin responses (cf. Pickles et.al., 1966) will be measured by the simultaneous addition of subthreshold doses of prostaglandins and oxytocin and measuring the contractile response. The effect of nicotine on these various parameters will be assesed by repeating these measurements with the muscle exposed to nicotine (40 - 80 µg/ml).

Measurement of the effect of nicotine on the dose-response relation of trachealis to TEA will also involve planimetry of phasic myograms; however, because desensitization of trachealis to TEA is not observed, cumulative dose-response relations will be measured. In other respects the experimental protocol for these studies is similar to that described above.

4. Stimulus - Response relation

Since responses to electrical stimulation are 95% inhibited by atropine (10^{-7}M), an effect of nicotine on neural elements should be evident in a shift of the stimulus-response relation. These effects will be measured by applying various intensities of electrical fields to muscles pre equilibrated at optimal length. Since the trachealis responds tonically to electrical stimulation the maximum isometric tension will be taken as the response and plotted as a function of the stimulus intensity. These studies will be performed in the presence and absence of atropine and nicotine.

B. Biochemical Studies

1. Measurement of prostaglandin release

The methods used will be essentially those developed by Vane and Williams (1973)

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APPENDIX 2 CONT'D

and Gilmore et.al. (1968) for the extraction and assay of prostaglandins and Green and Samuelson (1964) for the separation of these compounds by thin-layer chromatograph.

After exposure of the bathing solution to the muscle (or decidua) in the presence and absence of nicotine, the solution is collected and the prostaglandins extracted. The latter is accomplished by acidifriction of the solution to pH3 with IN HCl and extraction twice with one volume ethyl acetate. The ethyl acetate phases are pooled and evaporated to dryness at 40°C in the presence of a vacuum. The residue is stored at -12°C until required for assay.

Bioassay for prostaglandin-like activity is done using superfused strips of rat stomach and rat colon in the presence of a variety of antagonists to block responses to other agents which may be present in the extract. Specifically, responses to acetylcholine, 5-hydroxytryptamine, histamine and catecholamines are blocked by the addition of hyosine hydrobromide (10^{-7} g/ml) methysergide maleate (2×10^{-7} g/ml) mepyramine maleate (10^{-7} g/ml) phenoxybenzamine hydrochoride (10^{-7} g/ml) and propranolol hydrochloride (10^{-6} g/ml). Since the rat stomach is about ten times as sensitive to PGE_2 than to $PGF_{2\alpha}$ whereas the rat colon is equally sensitive to the two agents bioassay is accomplished by exposure of these preparations to standard amounts of these prostaglandins and to the muscle extracts. The results of the initial bioassay are confirmed by separating the prostaglandins in the extract by thin-layer chromatography and re-assay. Specifically, a part of the extract is taken up in 0.5 ml absolute ethanol and applied, alongside standard prostaglandins, to glass plates pre-coated with 0.25 mm. silica gel. Plates are developed in the AI solvent system of Green and Samuelson (1964) which contains benzene, dioxane and acetic acid in the ratio 20:20:1. When the solvent front nears the top, the plates are dried at room temperature and the substances visualized by spraying with water. The areas corresponding to the authentic prostaglandins (as well as intermediate areas) are then scraped off into separate tubes, taken up in Krebs-Ringer solution, filtered, and assayed as described previously. Recovery is estimated by including tritium-labelled prostaglandin standards.

2. Measurement of tissue cyclic AMP content

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(a) Muscle equilibration

Strips of term pregnant myometrium are mounted isometrically on glass rods and pre-equilibrated in normal Krebs-Ringer solution as described above. The effect of nicotine on cAMP levels and the response to β-adrenoceptor activation (by isoproterenol, 10^{-6}M) will be measured by equilibrating paired muscle in the presence and absence of nicotine and in in the presence of isoproterenol and isoproterenol + nicotine (40 - 80 µg/ml). At the appropriate time (i.e. at the time of initial and maximal physiological response) muscles are rapidly withdrawn and plunged into liquid N_2. Another protocol which has provided a very sensitive index of changes in the breakdown of cAMP in whole tissue is one in which the tissue cAMP content is first increased by prior exposure to isoproterenol and then the decrease in tissue cAMP monitored during the interval immediately following in which isoproterenol is withdrawn and α- and β-adrenoceptor blocking agents introduced. Since diverse agents which cause contraction have been shown to increase the breakdown of cAMP (Kroeger and Moorhouse, 1974) the effect of nicotine (40 - 80 µg/ml) will be tested in the proposed studies. Specifically, the effect of nicotine will be compared with untreated controls and other

(Continued on page 2f)

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APPENDIX 2 CONT'D

paired experimental muscle exposed to nicotine and hexamethonium, atropine, propranolol or phentolamine, the latter being designed to differentiate between direct effects on the muscle and nerve-mediated effects.

(b) Biochemical analysis

Frozen tissues are pulverized in the presence of 0.5 M $HClO_4$, extracted at 37° for 4 minutes and centrifuged (2000 g x 2 min.). A triethamolamine - KCl buffer, pH 7.5 is added to the supernatant, the mixture neutralized (pH = 7.5) and centrifuged (2000 g x 4 min.). Aliquots of the supernatant are transferred to small vials and lyophilized. These are then assayed for cAMP by a protein binding assay (obtained in kit form from Amersham-Searle). In this method the experimental samples and "standards" are equilibrated with ^3H cAMP and a cAMP binding protein in the presence of a buffer. At the end of this equilibration (90 min.) activated charcoal is added to all tubes, (to remove unbound ^3H cAMP) the mixture is thoroughly mixed (Vortex, 10 sec) and centrifuged (2000 g x 4 min.). An aliquot of the supernatant is transferred to a scintillation cocktail and counted for ^3H. Thus the number of counts obtained is inversely related to the non-radioactive cAMP present in the tube. The standard curve obtained (after subtraction of appropriate blanks) is linear. This method has proved to be very sensitive, reproducible and free from artefacts in our hands.

C. Electrophysiological Studies

1. Measurement of Membrane potential

The segments are mounted in a 10-ml organ bath maintained at 37 C and arranged for the recording of membrane potentials and tension. Glass microelectrodes of 30-80 MΩ resistance filled with 2 m KCl are used to penetrate the smooth muscle cells. Membrane potentials and tension were displayed on a dual-beam oscilloscope and photographed on 35 mm film. The rate of flow of solution through the organ bath is about 5 ml/min.

The general procedure is to equilibrate the segments in normal solution for 1-2 hr. During equilibration the resting tension (residual tension between spontaneous contractions) is gradually adjusted to about 0.2 g by means of a micromanipulator to which the strain gauge is attached. After equilibration is complete measurements are made of resting membrane potential (maximum level of membrane polarization between spontaneous contractions), action potential and frequency during contraction. The electrical records are accepted only if the electrode remains in the same cell before, during, and after one or more spontaneous contractions. Additional criteria for an acceptable electrode penetration are: 1) an abrupt shift from the extracellular reference potential to a negative resting potential when the electrode enters the cell; 2) a stable resting potential of the same magnitude before and after a spontaneous contraction, 3) action potentials of uniform height and of magnitude equal to or greater than the absolute value of the resting potential, 4) no change in reference potential, electrode resistance, or tip potential after withdrawal of the electrode from the cell.

While the electrode remains within the same cell from which the control measurements are made, the test substance (nicotine) is added to the bathing medium and the resultant effects on membrane potential and contraction are measured. Only those experiments in which the microelectrode remains in the same cell during control and test periods are taken for analysis.

The term membrane potential refers to the potential measured by an intracellular electrode

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APPENDIX 2 CONT'D

relative to the extracellular fluid during a quiescent period between spontaneous contractions and hence the resting membrane potential has a negative sign.

2. Measurement of Electrotonic Potentials

The muscle strip is placed in a muscle chamber similar to that described by Abe and Tomita (1968). In this chamber polarizing current pulses are applied to the tissue in the direction of the long axis of the muscle fibers by means of two silver-silver chloride plate electrodes 1 cm apart. One of the stimulating electrodes serves as a partition and divides the chamber into two compartments, one for stimulating and one for recording. The muscle is passed through a small hole in the partition electrode so that about one-third of the tissue is in the stimulating bath and the remainder in the recording bath. The surface of the electrode facing the recording chamber is coated with araldite. Both chambers are irrigated independently with physiological salt solution at 37 C and gassed with 95% O_2-5% CO_2.

The magnitude of the applied voltage gradient is recorded with silver-silver chloride electrodes, 5 mm apart, placed in the middle of the stimulating bath. The current passing through the tissue is assumed to be directly proportional to the applied voltage gradient. Rectangular pulses (600 msec duration, variable intensity) are delivered to the plate electrodes from a Grass S-4 stimulator. Stimuli are isolated from ground and passed through a load resistor (39 KΩ) to obtain a constant current.

Electrotonic potentials are elicited by electrical stimulation of the muscle during quiescent periods between contractions and are recorded with intracellular microelectrodes in cells located about 1 mm from the partition electrode. Only those experiments in which the electrode remains in the same cell, and hence at the same distance from the partition electrode before and during the action of the test agents are accepted for analysis.

Since the decay of electrotonic potentials is inversely related to membrane resistance these measurements yield evidence concerning the effect of the test drug on membrane permeability (Kroeger, and Marshall, 1973). Also, by determining the current (applied)-voltage (electrotonic potential) relationship at different measured distances from the stimulating electrode the effect of nicotine on the length constant can be determined. By using the stimulating electrodes to elicit action potentials and monitoring these at various measured distances the effect of nicotine on conduction velocity can also be measured. Rectification, a measure of the increase in membrane permeability to K^+ with depolarization as evidenced by a flattening of the current-voltage relation with positive electrotonic potentials will also be investigated as a possible site of action of nicotine on the muscle membranes. Thus these techniques yield a variety of information and permit a thorough analysis of the effect of nicotine on the electrophysiological parameters of the muscle cell membrane.

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D. Estimate of time required for these studies

Since all of the techniques and methods proposed (with the exception of prostaglandin assays) are in routine use in my laboratory, (see reprints attached) there should be a minimum of time required for commencing these studies. (The prostaglandin assays require only slight modifications of procedures already in use here and I do not anticipate major delays in performing this aspect of the study.) On the basis of my past experience with studies of this kind, the following represents what I consider a realistic budget of the time required for the various aspects of this study:

(Continued on page 2h)

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APPENDIX 2 CONT'D

Year 1: (Myometrium) - All biophysical studies on myometrium
- All cyclic AMP assays
- Prostaglandin assays set up and standardized

Year 2: (Myometrium) - Prostaglandin assays completed
- Electrophysiological studies

Year 3: (Trachealis) - All studies proposed to be completed

The above estimate is based on the assumption that funds for a full-time technician and student are approved. Since these studies involve a variety of interrelated investigative techniques on an important physiological problem this would appear to be a good project on which to train a graduate student.

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APPENDIX 2 CONT'D

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KROEGER, E.A. and J.M. MARSHALL, (1974). Beta-adrenergic effects on rat myometrium: Role of cyclic AMP. Am. J. Physiol. (In Press).

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(Continued on page 2j)

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Source: https://www.industrydocuments.ucsf.edu/docs/ggdm0000

APPENDIX 2 CONT'D

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STEPHENS, N.L. and E. KROEGER, (1970). Effect of hypoxia on airway smooth muscle mechanics and electrophysiology. J. Appl. Physiol. 28: 630-635.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

My laboratory comprises about 600 square feet of normal laboratory space plus a shielded room for electrophysiological studies which I share with Dr. N. L. Stephens. My present facilities include apparatus for measuring and recording the biophysical properties of muscle (constant temperature organ baths, strain guages, amplifiers and recorder), biochemical apparatus for measuring cyclic AMP(shaker bath, freeze dryer, fluorimeter) and electrophysiological equipment (including a Tomita bath, oscilloscope, 35 mm. camera, electrometer, stimulator and associated calibrating equipment). In addition I have access to preparative and ultracentrifuges, scintillation counters, a cold room, glassware washer, electronics shop and computer terminal which are shared departmental facilities. Thus, while some equipment will have to be purchased in order to accommodate the additional work load of the proposed studies, the space and major facilities are adequate for this work.

11. Additional facilities required:

Since my present apparatus for biophysical studies is used to capacity in ongoing research, the extensive biophysical studies proposed will require the construction of another such apparatus with a capacity for four muscles (to allow paired control and experimental muscles i.e. one control for three experimental muscles as the protocols require). The equipment needed is as follows: one multi-channel recorder, four organ baths, force transducers and coupler-amplifiers and a circulating water bath.

My present equipment and facilities should be adequate for all other aspects of the proposed studies.

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12. Biographical sketches of investigator(s) and other professional personnel (append): (See Appendix 3)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):
(See Appendix 4)

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R: REDACTED MATERIAL

APPENDIX 3

12. PERSONAL DATA

Name: KROEGER, Edwin A.

Date of Birth: REDACTED

Marital Status:

Citizenship: REDACTED

Education:

Wheaton College	B.Sc.	1966	Biology
University of Manitoba	M.Sc.	1968	Physiology
University of Manitoba	Ph.D.	1970	Physiology

Positions Held:

1972 -	Scholar of the MRC, Assistant Professor, Department of Physiology, Faculty of of Medicine, University of Manitoba
1971 - 72	Visiting Instructor, Division of Bio-Med Sciences, Brown University
1970 - 71	Research Associate, Division of Bio-Med Science, Brown University
1967 - 70	Demonstrator, Department of Physiology, Faculty of Medicine, University of Manitoba

Participation in Research

1966-1968, Department of Physiology, University of Manitoba

Studies on effect of hypoxia on mechanical properties and electrophysiology of tracheal smooth muscle (under the supervision of Dr. N.L. Stephens). These studies were done in partial fulfillment of the requirements for the M.Sc. degree. Although the title of the project was suggested, its development, execution and adaptation of techniques were my responsibility. In particular, the development of electrophysiological techniques for the study of this muscle required considerable effort since the trachealis has a considerable amount of connective tissue, small diameter muscle fibers, and proved to be electrically quiescent. These characteristics made it difficult not only to penetrate smooth muscle cells but also to ascertain that the electrode was in a cell at a given moment, since pressure artifacts can often mimic cell impalements in causing a change in recorded potential. In the preliminary investigations therefore, to validate the recording characteristics of the instrumentation, I showed that 1) the normal electrical activity of the well-documented smooth muscle preparations could be recorded, and 2) on impalement of trachealis cells, a depolarization could be recorded on increasing the extracellular K^+ concentration.

1968-1970, Department of Physiology, University of Manitoba

Studies on effect of hypoxia on energy and calcium metabolism in tracheal smooth muscle (under the supervision of Dr. N.L. Stephens, in partial fulfillment of the requirements for the Ph.D. degree). In attempting to gain a deeper insight into the mechanisms causing the mechanical effects of hypoxia, it was apparent that this would require a careful definition of cellular energy and calcium metabolism. Since such studies had not been undertaken previously in that laboratory this

(Continued on page 3b)

10035446 17

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APPENDIX 3 CONT'D

Participation in Research Cont'd.

required the setting up of techniques for the measurement of glycogen, ATP, ADP, AMP, creatine phosphate, Ca^{45} efflux and tissue Ca. In addition our requirement that these parameters be measured in muscles on which we had detailed mechanical information confirming the effect of hypoxia necessitated the modification of these procedures for the small muscles used in mechanical studies. The development and execution of these measurements was my responsibility.

1970-1972, Division of Biological and Medical Science, Brown University, Providence, R.I.

Studies on the mechanism of hyperpolarization and relaxation of uterine smooth muscle in response to beta-adrenergic agents (as a Research Associate of Dr. J.M. Marshall and, Visiting Instructor of Biological and Medical Sciences). Electrophysiological techniques are well established in this laboratory. In the measurement of cyclic AMP, I was fortunate in being allowed the use of Dr. John Fain's facilities. This did, however, require modifications so that the relatively small amounts of this substance found in smooth muscle could be measured. In the measurement of tissue Ca the utility of the relatively new "La-technique" was demonstrated and exploited for demonstrating the small but significant changes in tissue Ca which may be responsible for relaxation. The development and execution of these measurements in this laboratory were my responsibility. These findingswere pursued in collaboration with Dr. C. Paul Bianchi who allowed me the use of his laboratory for the "pulse-labelling" studies. His tutelage on the interpretation of ion flux measurements was invaluable.

1972-1974, Department of Physiology,

During these past years, as a Scholar of the MRC and Assistant Professor of Physiology, I have set up a laboratory in which biophysical, electrophysiological and biochemical studies are currently in progress and have conducted the research on mechanisms of tone in smooth muscle. This has included a documentation of 1) the length-dependency of basal tone in myometrium and its modification by various ionic conditions, 2) the influence of TEA on tone, myogenic response and the electrophysiological properties of tracheal smooth muscle, 3) the cyclic AMP content of myometrium in various ionic and hormonal conditions and 4) the oxygen consumption of myometrium and the effect of various agents which modify tone.

Publications:

Abstracts:

1. Stephens, N.L., E. Kroeger and A. Naimark (1968). Force-velocity relationships of canine tracheal smooth muscle: Effect of hypoxia. Fed. Proc. 27 (2): 228.

2. Stephens, N.L., E. Kroeger and U. Kromer. Properties of airway smooth muscle (Trachealis) mitochondria. P0940 of Abstracts of presentations at the 12th Annual Meeting of the Canadian Thoracic Society, June 1970.

3. Marshall, J.M. and E. Kroeger. Beta-adrenergic effects on rat uterus. 1. Ionic mechanisms of hyperpolarization. The Pharmacologist 13: 315, 1971.

(Continued on page 3c)

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APPENDIX 3 CONT'D

Abstracts Cont'd

4. Kroeger, E. and J.M. Marshall. Beta-adrenergic effects on rat uterus. 2. Relation to cAMP and Ca. The Pharmacologist 13: 315, 1971.

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7. Kroeger, E., J. Marshall and C. Biancki. Effects of Muscle relaxants on tissue Ca in the rat myometrium. Fed. Proc. 32: 767, 1973.

8. Kroeger, E.A. and J.A. Moorhouse. Initiation of phasic mechanical and electrical activity in tonic tracheal airway smooth muscles (TSM) with tetraethylammonium (TEA^+). Fed. Proc. 32: 389, 1973.

Papers

1. Stephens, N.L., E. Kroeger and J.A. Mehta. Force-velocity characteristics of respiratory airway smooth muscle. J. Appl. Physiol. 26 (6): 685-692, 1969.

2. Stephens, N.L. and E. Kroeger. Effect of hypoxia on airway smooth muscle mechanics and electro-physiology. J. Appl. Physiol. 28 (5): 630-635, 1970.

3. Kroeger, E. and N.L. Stephens. Effect of hypoxia on energy and calcium metabolism in airway smooth muscle. Amer. J. Physiol. 220 (5): 1199-1204, 1971.

4. Stephens, N.L., E.A. Kroeger and U. Kromer. Induction of myogenic response in tonic airway smooth muscle by tetraethylammonium. Amer. J. Physiol. (In Press)

5. Kroeger, E.A. and N.L. Stephens. Effect of tetraethlammonium on tonic airway smooth muscle: Initiation of phasic electrical activity. Amer. J. Physiol. (In Press)

6. Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Mechanisms of membrane hyperpolarization. Amer. J. Physiol. 225: 1339-1345.

7. Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Role of cyclic AMP. Amer. J. Physiol. (In Press)

8. Kroeger, E.A., J.M. Marshall, and C.P. Bianchi. Effect of isoproterenol and D-600 on Ca movements in rat myometrium. Submitted to J. Pharmacol. Exp. Therap.

9. Marshall, J.M. and E.A. Kroeger. Adrenergic influences on uterine smooth muscle. Phil. Trans. Roy. Soc. Lond. B 265: 135-148, 1973.

10035446 19

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APPENDIX 4

3. PUBLICATIONS:

Stephens, N.L., E. Kroeger and J.A. Mehta. Force-velocity characteristics of respiratory airway smooth muscle. J. Appl. Physiol. 26 (6): 685-692, 1969.

Kroeger, E. and N.L. Stephens. Effect of hypoxia on energy and calcium metabolism in airway smooth muscle. Amer. J. Physiol. 220 (5): 1199-1204, 1971.

✓ Kroeger, E.A. and N.L. Stephens. Effect of tetraethlammonium on tonic airway smooth muscle: Initiation of phasic electrical activity. Amer. J. Physiol. (In Press)

Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Mechanisms of membrane hyperpolarization. Amer. J. Physiol. 225: 1339-1345.

Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Role of cyclic AMP. Amer. J. Physiol. (In Press).

Marshall, J.M. and E.A. Kroeger. Adrenergic influences on uterine smooth muscle. Phil. Trans. Roy. Soc. Lond. B 265: 135-148, 1973.

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R: REDACTED MATERIAL

14. First year budget:

	% time	Amount
A. Salaries (give names or state "to be recruited")		
Professional (give % time of investigator(s) even if no salary requested)		
E. A. Kroeger		REDACTED
Student - to be recruited		
Technical		
One experienced technician - to be recruited		REDACTED
Sub-Total for A		R
B. Consumable supplies (by major categories)		
Animals - timed pregnant rats, 200 ($8.00 each)		1,600
- boarding charges 40 (.03/diem)		428
Chemicals - for physiological salt solutions, scintillation counting cocktails, biochemical assays		1,800
Glassware and disposable supplies		200
Gas mixtures (for equilibrating physiological salt solutions)		100
Chart paper (for recorder - 6 rolls ($8.40 each)		50.40
Sub-Total for B		$ 4,178.40
C. Other expenses (itemize)		
Equipment maintenance		200
Glassware washer (1 hour /day at $2.00)		520
Sub-Total for C		720
Running Total of A + B + C		$16,898.40
D. Permanent equipment (itemize).		
Oscillograph recorder (see justification above #11) Brush, Mark 440 - 4 channel		3,350.
Amplifier (4) and transducer couplers (4) Brush ($775.00 each)		3,100
Organ baths (4) ($50.00 each)		200
Circulating water bath		345
Force transducers (4): Statham ($180.00 each)		720
Rack and pinion assemblies (4) ($90.00 each)		360
Sub-Total for D		$ 8,075
E		$ 2,534.76
Total request		$27,508.16

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	13,000	5,000	1,000	1,700	2,850	23,550
Year 3	14,000	2,500	600	-	2,565	19,665

10035446 21

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The mechanism of production and regulation of tone in mammalian smooth muscle: Biophysical, electrophysiological and biochemical studies	Medical Research Council MA 4765	$ 8,400	April 1, 1974 March 31, 1975.
The effect of cyclic AMP on Ca transport of plasma membrane of pregnant myometrium	Banting Research Foundation	7,200	July 1, 1974 June 30, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Role of Ca & Mg in the biophysical and electrophysiological response of tracheal smooth muscle to tetraethylammonium	Medical Research Council	15,000	April 1, 1975 March 31, 1976.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name Edwin A. Kroeger

Signature Edwin C. Kroeger Date July 29, 1974

Telephone 204 786-3733
Area Code Number Extension

Checks payable to
University of Manitoba

Mailing address for checks
Comptroller's Office, The University of Manitoba
Winnipeg, Manitoba, Canada

Responsible officer of institution

Typed Name R.A. Lebrun

Title Executive Assistant to the President

Signature [illegible] Date JUL 30 1974

Telephone 204 474-8308
Area Code Number Extension

10035446 22

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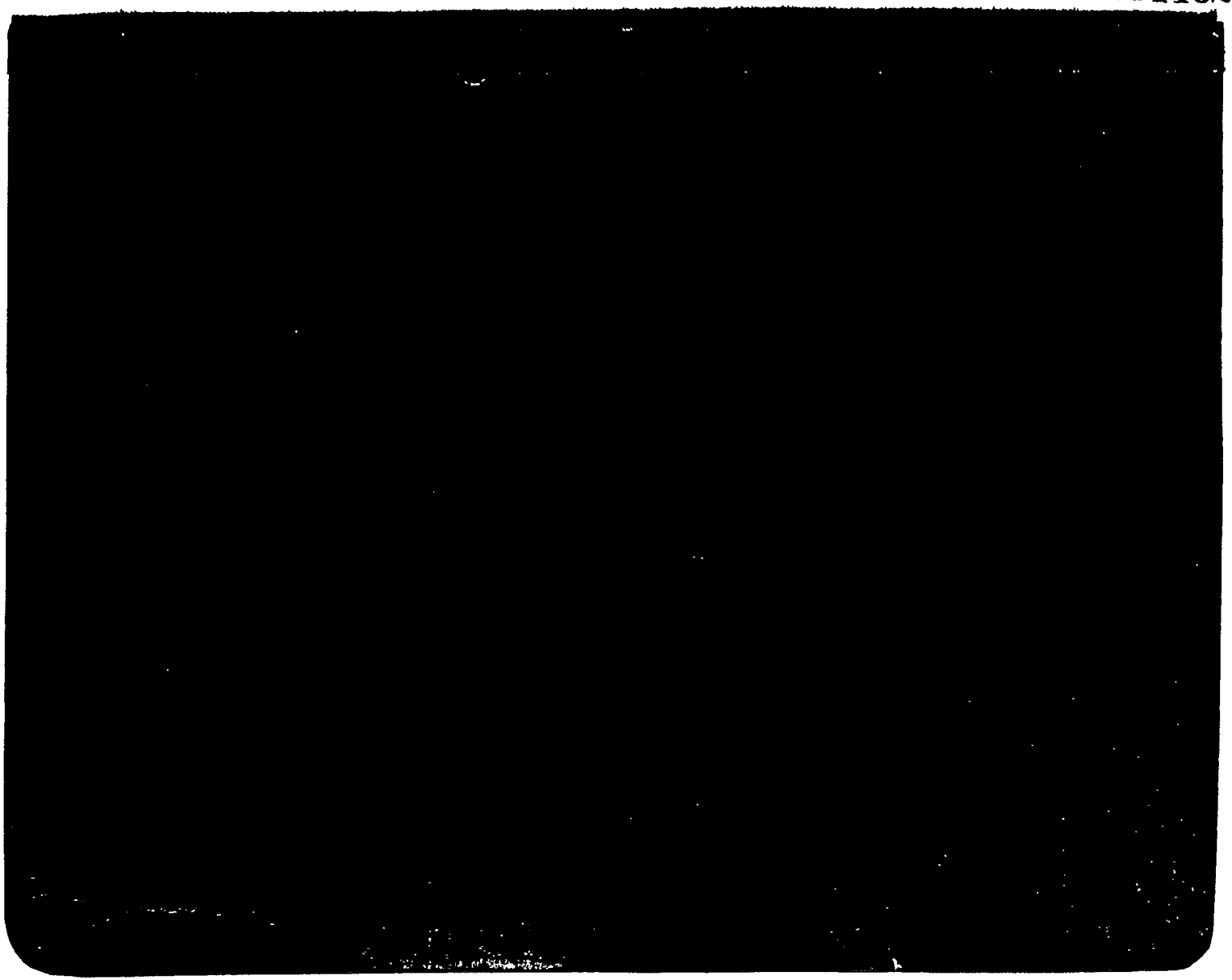

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